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(54) Title: BROAD SPECTRUM TUMOR SUPPRESSOR GENES, GENE PRODUCTS AND METHODS FOR TUMOR SUPPRESSION GENE THERAPY (57) Abstract <p>The present invention relates to a broad-spectrum tumor suppressor gene and the protein expressed by that gene in appropriate hostcells. The protein is a second in-frame AUG codon-initiated retinoblasoma protein of about 94 kD relative molecular mass. The present invention also relates to methods of treating a mammal having a disease or disorder characterized by abnormal cellular proliferation, such as a tumor or cancer and methods of treating abnormally proliferating cells, such as tumor or cancer cells. Treatment is accomplished by inserting a host cell compatible p94^{RB} expression vector or an effective amount of p94^{RB} protein into a cell or cells in need of treatment.</p>		

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**BROAD SPECTRUM TUMOR SUPPRESSOR GENES, GENE PRODUCTS AND METHODS
FOR TUMOR SUPPRESSION GENE THERAPY**

5 This invention was made in part with United States government support under grant number EY06195 awarded by National Institutes of Health. The United States government has certain rights in the invention.

1. BACKGROUND OF THE INVENTION

10 1.1 Field of the Invention

This invention is in the field of tumor suppressor genes (anti-oncogenes) and relates in general to products and methods for practicing broad-spectrum tumor suppressor gene therapy of various human cancers. In particular, the invention relates to methods for treating tumor cells (1) administering vectors comprising a nucleic acid sequence coding for a second in-frame AUG codon-initiated retinoblastoma protein of about 94 kD or (2) administering an effective amount of a protein coded for by the nucleic acid sequence.

1.2 Cancer

25 Cancers and tumors are the second most prevalent cause of death in the United States, causing 450,000 deaths per year. One in three Americans will develop cancer, and one in five will die of cancer (Scientific American Medicine, part 12, I, 1, section dated 1987). While substantial progress has been made in identifying some of the likely environmental and hereditary causes of cancer, the statistics for the cancer death rate indicates a need for substantial improvement in the therapy for cancer and related diseases and disorders.

1.3. Cancer Genes

A number of so-called cancer genes, i.e., genes that have been implicated in the etiology of cancer, have been identified in connection with hereditary
5 forms of cancer and in a large number of well-studied tumor cells. Study of cancer genes has helped provide some understanding of the process of tumorigenesis. While a great deal more remains to be learned about cancer genes, the presently known cancer genes serve
10 as useful models for understanding tumorigenesis.

Cancer genes are broadly classified into "oncogenes" which, when activated, promote tumorigenesis, and "tumor suppressor genes" which, when damaged, fail to suppress tumorigenesis. While
15 these classifications provide a useful method for conceptualizing tumorigenesis, it is also possible that a particular gene may play differing roles depending upon the particular allelic form of that gene, its regulatory elements, the genetic background
20 and the tissue environment in which it is operating.

1.3.1. Oncogenes

The oncogenes are somatic cell genes that are mutated from their wild-type alleles (the art refers
25 to these wild-type alleles as protooncogenes) into forms which are able to induce tumorigenesis under certain conditions. There is presently a substantial literature on known and putative oncogenes and the various alleles of these oncogenes. In order to
30 provide background information and to further the understanding of the scope of the invention, a brief discussion of representative oncogenes is provided.

For example, the oncogenes *ras* and *myc* are considered as models for understanding oncogenic
35 processes in general. The *ras* oncogene is believed to encode a cytoplasmic protein, and the *myc* oncogene is believed to encode a nuclear protein. Neither the *ras*

oncogene nor the *myc* oncogene alone is able to induce full transformation of a normal cell into a tumor cell, but full tumorigenesis usually occurs when both the *ras* and *myc* oncogenes are present and expressed together in the same cell (Weinberg, R.A., 1989, Cancer Research 49:3713-3721, at page 3713). Such collaborative effects have been observed between a number of other studied oncogenes.

The collaborative model of oncogene tumorigenesis must be qualified by the observation that a cell expressing the *ras* oncogene that is surrounded by normal cells does not undergo full transformation. However, if most of the surrounding cells are also *ras*-expressing, then the *ras* oncogene alone is sufficient to induce tumorigenesis in a *ras*-expressing cell. This observation validates the multiple hit theory of tumorigenesis because a change in the tissue environment of the cell hosting the oncogene may be considered a second hit.

An alternative and equally valid hypothesis is that events that collaborate with the activation of an oncogene such as *ras* or *myc* may include the inactivation of a negative regulatory factor or factors (Weinberg, R.A., 1989, Cancer Research 49:3713-3721, at 3717; Goodrich, D.W. and Lee, W-H., 1992, Nature 360:177-179), i.e., a tumor suppressor protein.

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1.3.2. Tumor Suppressor Genes

Tumor suppressor genes are genes that, in their wild-type alleles, express proteins that suppress abnormal cellular proliferation. When the gene coding for a tumor suppressor protein is mutated or deleted, the resulting mutant protein or the complete lack of tumor suppressor protein expression may fail to correctly regulate cellular proliferation, and abnormal cellular proliferation may take place, particularly if there is already existing damage to the cellular regulatory mechanism. A number of well-studied human tumors and tumor cell lines have been shown to have missing or nonfunctional tumor suppressor genes. Examples of tumor suppression genes include, but are not limited to, the retinoblastoma susceptibility gene or RB gene, the p53 gene, the deleted in colon carcinoma (DCC) gene and the neurofibromatosis type 1 (NF-1) tumor suppressor gene (Weinberg, R.A. Science, 1991, 254:1138-1146). Loss of function or inactivation of tumor suppressor genes may play a central role in the initiation and/or progression of a significant number of human cancers.

The list of putative tumor suppressor genes is large and growing. The following discussion of tumor suppressor genes is not intended to provide a complete review of all known and putative tumor suppressor genes, but is provided as background to indicate the state of the art and the problems to be overcome before the art is able to provide successful genetic therapy of diseases and disorders characterized by abnormally proliferating cells, e.g., tumor or cancer cells.

1.3.2.1. The Retinoblastoma Gene

The RB gene is one of the better studied tumor suppressor genes. The size of the RB gene complementary DNA (cDNA), about 4.7 Kb, permits ready

manipulation of the gene, so that insertions of the RB gene have been made into a number of cell lines. The RB gene has been shown to be missing or defective in a majority of retinoblastomas, sarcomas of the soft
5 tissues and bones, and in approximately 20 to 40 percent of breast, lung, prostate and bladder carcinomas (Lee, W-H., et al., PCT Publ. No. WO 90/05180, at pages 38 and 39; see also, Bookstein, R. and Lee, W-H., 1991, Crit. Rev. Oncog., 2:211-217;
10 Benedict, W.F. et al., J. Clin. Invest., 1990, 85:988-993).

Based upon study of the isolated RB cDNA clone, the predicted RB gene product has 928 amino acids and an expected molecular weight of 106 kD (Lee et al.,
15 1987, Nature, 329:642-645). The natural factor corresponding to the predicted RB gene expression product has been identified as a nuclear phosphoprotein having an apparent relative molecular mass (Mr) of 110-114 kD (Lee et al., 1987, Nature,
20 329:642-645) or 110-116 kD (Xu et al., 1989, Oncogene 4:807-812). Hence, the literature generally refers to the protein encoded by the RB gene as p110^{RB}. In this connection, it is noteworthy that measurement of
apparent relative molecular mass by SDS-PAGE is
25 frequently inaccurate owing to protein secondary structure. Therefore, the full length RB protein of 928 amino acids is also referred to as the 115 kD (Yokota et al., 1988, Oncogene, 3:471-475), or 105 kD (Whyte et al., 1988, Nature, 334:124-129) RB proteins.
30 Various mutations of the RB gene are known. These are generally inactive. However, a 56 kD truncated RB protein, designated as p56^{RB}, that is considered to function in the same way as does p110^{RB} retains activity (Goodrich et al., 1992, Nature 360:177-179).

35 On SDS-PAGE normal human cells show an RB protein pattern consisting of a lower sharp band with an Mr of 110 kD and a broader, more variable region above this

band with an Mr ranging from 110 kD to 116 kD. The 110 kD band is the underphosphorylated RB protein, whereas the broader region represents the phosphorylated RB protein. The heterogeneity of the
5 molecular mass results from a varying degree of phosphorylation (Xu et al., 1989, Oncogene, 4:807-812).

The RB protein shows cyclical changes in phosphorylation. Most RB protein is unphosphorylated
10 during G1 phase, but most (perhaps all) RB molecules are phosphorylated in S and G2 phases (Xu et al., 1989, Oncogene, 4:807-812; DeCaprio et al., 1989, Cell, 58:1085-1095; Buchkovich et al., 1989, Cell, 58:1097-1105; Chen et al., 1989, Cell, 58:1193-1198;
15 Mihara et al., 1989, Science, 246:1300-1303). Furthermore, only the underphosphorylated RB protein binds to SV40 large T antigen. Given that RB protein binding by large T antigen is probably important for the growth promoting effects of large T antigen, this
20 suggests that the underphosphorylated RB protein is the active form of the RB protein, and the phosphorylated RB protein in S and G2 phases is inactive (Ludlow et al., 1989, Cell, 56:57-65).

The RB gene expressing the first in-frame AUG
25 codon-initiated RB protein is also referred to herein as the intact RB gene, the RB¹¹⁰ gene or the p110^{RB} coding gene. It has also been observed that lower molecular weight (<100 kD, 98 kD, or 98-104 kD) bands of unknown origin which are immunoreactive to various
30 anti-RB antibodies can be detected in immunoprecipitation and Western blots (Xu et al., 1989, Oncogene, 4:807-812; Furukawa et al., 1990, Proc. Natl. Acad. Sci., USA, 87:2770-2774; Stein et al., 1990, Science, 249:666-669).

35 Considering that the RB¹¹⁰ cDNA open reading frame sequence (McGee, T.L., et al., 1989, Gene, 80:119-128) reveals an in-frame second AUG codon located at exon

3, nucleotides 355-357, the deduced second AUG codon-initiated RB protein would be 98 kD, or 12 kD smaller than the p110^{RB} protein. It has been proposed that the lower molecular weight bands are the

5 underphosphorylated (98 kD) and phosphorylated (98-104 kD) RB protein translated from the second AUG codon of the RB mRNA (Xu et al., 1989, Oncogene, 4:807-812), although no data directly supported this hypothesis. Thus, no conclusive observation confirms the actual

10 expression of the RB gene from the second in-frame AUG codon. Further, Sections 4.2.1, and Figure 5 *infra* provide data indicating the non-identity of the 98 kD protein bands of unknown origin and the second AUG codon-initiated protein products.

15 It has been proposed that introduction of a functional RB¹¹⁰ gene into an RB-minus tumor cell will likely "normalize" the cell. Of course, it is not expected that tumor cells which already have normal RB¹¹⁰ gene expression ("RB+") will respond to RB¹¹⁰ gene

20 therapy, because it is presumed that adding additional RB expression cannot correct a non-RB genetic defect. In fact, it has been shown that in the case of RB+ tumor cell lines, such as the osteosarcoma cell line, U-2 OS, which expresses the normal p110^{RB}, introduction

25 of an extra p110^{RB} coding gene did not change the neoplastic phenotype of such tumor lines (Huang, et al., 1988, Science, 242:1563-1566).

In the only reported exception, introduction of a p110^{RB} coding vector into normal human fibroblasts,

30 WS1, which have no known RB or any other genetic defects, led to the cessation of cell growth (WO 91/15580, Research Development Foundation, by Fung et al., PCT application filed 10 April 1991, published 17 October 1991, at page 18). However, it is believed

35 that these findings were misinterpreted since a plasmid, ppVUO-Neo, producing SV40 T antigen with a

well-known growth-promoting effect on host cells was used improperly to provide a comparison with the effect of RB¹¹⁰ expression on cell growth of transfected WS1 fibroblasts (Fung, et al. Id. see Example 2 page 25). This view is confirmed by the extensive literature, together with similar confirming data provided by the examples presented *infra*, clearly characterizing RB+ tumor cells as "incurable" by treatment with wild-type RB¹¹⁰ gene. In addition, it is noteworthy that the WS1 cell line per se is a generally recognized non-tumorigenic human diploid fibroblast cell line with limited cell division potential in culture. Therefore, W091/15580 simply does not provide any method for effectively treating RB+ tumors with an RB¹¹⁰ gene. Thus, there remains a need for a broad-spectrum tumor suppressor gene for treating abnormally proliferating cells having any type of genetic defect.

1.3.2.2. The Neurofibromatosis Gene
Neurofibromatosis type 1 or von Recklinghausen neurofibromatosis results from the inheritance of a predisposing mutant allele or from alleles created through new germline mutations (C.J. Marshall, 1991, Cell, 64:313-326). The neurofibromatosis type 1 gene, referred to as the NF1 gene, is a relatively large locus exhibiting a mutation rate of around 10^{-4} . Defects in the NF1 gene result in a spectrum of clinical syndromes ranging from café-au-lait spots to neurofibromas of the skin and peripheral nerves to Schwannomas and neurofibrosarcomas.

The NF1 gene encodes a protein of about 2485 amino acids that shares structural similarity with three proteins that interact with the products of the ras protooncogene (Weinberg et al., 1991, Science, 254:1138-1146 at page 1141). For example, the NF1 amino acid sequence shows sequence homology to the

catalytic domain of ras GAP, a GTPase-activating protein for p21 ras (C.J. Marshall, 1991, Cell, 64:313-326 at pages 320 and 321).

5 The role of NF1 in cell cycle regulation is apparently a complex one that is not yet fully elucidated. For example, it has been hypothesized that it is a suppressor of oncogenically activated p21 ras in yeast (C.J. Marshall, (1991, Cell, 64:313-326, bridging pages 320 and 321, and citing to Ballester et
10 al, 1990, Cell, 63:851-859). On the other hand, other possible pathways for NF1 interaction are suggested by the available data (C.J. Marshall, 1991, Cell, 64:313-326 at page 321; Weinberg et al., 1991, Science, 254:1138-1146 at page 1141).

15 At present, no attempts to treat NF1 cells with a wild-type NF1 gene have been undertaken due to the size and complexity of the NF1 locus. Therefore, it would be highly desirable to have a broad-spectrum tumor suppressor gene able to treat NF1 and any other
20 type of cancer or tumor.

1.3.3.3. The p53 Gene

Somatic cell mutations of the p53 gene are said to be the most frequently mutated gene in human cancer
25 (Weinberg et al., 1991, Science, 254:1138-1146 at page 1143). The normal or wild-type p53 gene is a negative regulator of cell growth, which, when damaged, favors cell transformation (Weinberg et al. *supra*). As noted for the RB protein, the p53 expression product is
30 found in the nucleus, where it may act in parallel with or cooperatively with p110^{RB}. This is suggested by a number of observations, for example, both p53 and p110^{RB} proteins are targeted for binding or destruction by the oncoproteins of SV40, adenovirus and human
35 papillomavirus.

Tumor cell lines deleted for p53 have been successfully treated with wild-type p53 vector to

reduce tumorigenicity (Baker, S.J., et al., 1990, Science, 249:912-915). However, the introduction of either p53 or RB¹¹⁰ into cells that have not undergone lesions at these loci does not affect cell

5 proliferation (Marshall, C.J., 1991, Cell, 64:313-326 at page 321; Baker, S.J., et al., 1990, Science, 249:912-915; Huang, H.-J.S., et al., 1988 Science, 242:1563-1566). Such experiments suggest that

10 sensitivity of cells to the suppression of their growth by a tumor suppressor gene is dependent on the genetic alterations that have taken place in the cells. Such a dependency would be further complicated by the observation in certain cancers that alterations in the p53 tumor suppressor or gene locus appear after

15 mutational activation of the ras oncogene (Marshall, C.J., 1991, Cell, 64:313-326; Fearon, E.R., and Vogelstein, B., 1990, Cell, 61:759-767).

Therefore, there remains a need for a broad-spectrum tumor suppressor gene that does not depend on

20 the specific identification of each mutated gene causing abnormal cellular proliferation.

1.3.3.4. The Deleted in Colon Carcinoma Gene (DCC)

25 The multiple steps in the tumorigenesis of colon cancer are readily monitored during development by colonoscopy. The combination of colonoscopy with the biopsy of the involved tissue has uncovered a number of degenerative genetic pathways leading to the result

30 of a malignant tumor. One well studied pathway begins with large polyps of which 60% of the cells carry a mutated, activated allele of K-ras. A majority of these tumors then proceed to the inactivation-mutation of the gene referred to as the deleted in colon

35 carcinoma (DCC) gene, followed by the inactivation of the p53 tumor suppressor gene.

The DCC gene is a more than approximately one million base pair gene coding for a 190-kD transmembrane phosphoprotein which is hypothesized to be a receptor (Weinberg et al., 1991, Science, 254:1138-1146 at page 1141), the loss of which allows the affected cell a growth advantage. It has also been noted that the DCC has partial sequence homology to the neural cell adhesion molecule (Marshall, 1991, Cell, 64:313-326) which might suggest a role for the DCC protogene in regulating cell to cell interactions.

As can be appreciated, the large size and complexity of the DCC gene, together with the complexity of the K-ras, p53 and possibly other genes involved in colon cancer tumorigenesis demonstrates a need for a broad-spectrum tumor suppressor gene and methods of treating colon carcinoma cells which do not depend upon manipulation of the DCC gene or on the identification of other specific damaged genes in colon carcinoma cells.

20

1.4 Genetic Therapy: Gene Transfer Methods

The treatment of human disease by gene transfer has now moved from the theoretical to the practical realm. The first human gene therapy trial was begun in September 1990 and involved transfer of the adenosine deaminase (ADA) gene into lymphocytes of a patient having an otherwise lethal defect in this enzyme, which produces immune deficiency. The results of this initial trial have been very encouraging and have helped to stimulate further clinical trials (Culver, K.W., Anderson, W.F., Blaese, R.M., Hum. Gene. Ther., 1991, 2:107).

So far all but one of the approved gene transfer trials in humans rely on retroviral vectors for gene transduction. Retroviral vectors in this context are retroviruses from which all viral genes have been removed or altered so that no viral proteins are made

in cells infected with the vector. Viral replication functions are provided by the use of retrovirus 'packaging' cells that produce all of the viral proteins but that do not produce infectious virus.

- 5 Introduction of the retroviral vector DNA into packaging cells results in production of virions that carry vector RNA and can infect target cells, but no further virus spread occurs after infection. To distinguish this process from a natural virus
- 10 infection where the virus continues to replicate and spread, the term transduction rather than infection is often used.

The major advantages of retroviral vectors for gene therapy are the high efficiency of gene transfer

15 into replicating cells, the precise integration of the transferred genes into cellular DNA, and the lack of further spread of the sequences after gene transduction (Miller, A.D., Nature, 1992, 357:455-460).

- 20 The potential for production of replication-competent (helper) virus during the production of retroviral vectors remains a concern, although for practical purposes this problem has been solved. So far, all FDA-approved retroviral vectors have been
- 25 made by using PA317 amphotropic retrovirus packaging cells (Miller, A.D., and Buttimore, C., Molec. Cell Biol., 1986, 6:2895-2902). Use of vectors having little or no overlap with viral sequences in the PA317 cells eliminates helper virus production even by
- 30 stringent assays that allow for amplification of such events (Lynch, C.M., and Miller, A.D., J. Viral., 1991, 65:3887-3890). Other packaging cell lines are available. For example, cell lines designed for separating different retroviral coding regions onto
- 35 different plasmids should reduce the possibility of helper virus production by recombination. Vectors produced by such packaging cell lines may also provide

an efficient system for human gene therapy (Miller, A.D., 1992, Nature, 357:455-460).

Non-retroviral vectors have been considered for use in genetic therapy. One such alternative is the
5 adenovirus (Rosenfeld, M.A., et al., 1992, Cell, 68:143-155; Jaffe, H.A. et al., 1992, Nature Genetics 1:372-378; Lemarchand, P. et al., 1992, Proc. Natl. Acad. Sci. USA, 89:6482-6486). Major advantages of
10 adenovirus vectors are their potential to carry large segments of DNA (36 Kb genome), a very high titre (10^{11} ml⁻¹), ability to infect non-replicating cells, and suitability for infecting tissues in situ, especially in the lung. The most striking use of this vector so far is to deliver a human cystic fibrosis
15 transmembrane conductance regulator (CFTR) gene by intratracheal instillation to airway epithelium in cotton rats (Rosenfeld, M.A., et al., Cell, 1992, 63:143-155). Similarly, herpes viruses may also prove valuable for human gene therapy (Wolfe, J.H., et al.,
20 1992, Nature Genetics, 1:379-384). Of course, any other suitable viral vector may be used for genetic therapy with the present invention.

The other gene transfer method that has been approved by the FDA for use in humans is the transfer
25 of plasmid DNA in liposomes directly to human cells in situ (Nabel, E.G., et al., 1990, Science, 249:1285-1288). Plasmid DNA should be easy to certify for use in human gene therapy because, unlike retroviral vectors, it can be purified to homogeneity. In
30 addition to liposome-mediated DNA transfer, several other physical DNA transfer methods such as those targeting the DNA to receptors on cells by complexing the plasmid DNA to proteins have shown promise in human gene therapy (Wu, G.Y., et al., 1991, J. Biol. Chem.,
35 266:14338-14342; Curiel, D.T., et al., 1991, Proc. Natl. Acad. Sci. USA, 88:8850-8854).

1.5 Proposed Strategies for Cancer Gene Therapy

It has been observed that certain tumor cells return to normal function when fused with normal cells, suggesting that replacement of a missing
5 factor, such as a wild-type tumor suppressor gene expression product may serve to restore a tumor cell to a normal state (reviewed by Weinberg, R.A., 1989, Cancer Research 49:3713-3721, at 3717).

These observations have led to research aimed at
10 providing genetic treatment of tumor cells having defective tumor suppressor genes. The proposed method of treatment requires identification of the damaged tumor suppressor gene, and introduction of the corresponding undamaged gene (including a promoter and
15 a complete encoding sequence) into the affected tumor cells by means of a vector such as a retrovirus able to express the gene product. It is proposed that the incorporated functional gene will convert the target cell to a non-malignant state.

20 For example, The Regents of the University of California, in Patent Cooperation Treaty patent application (by Lee et al., number WO 90/05180, having an international filing date of 30 October 1989 and published 17 May 1990), disclose a scheme for
25 identifying an inactive or defective tumor suppressor gene and then replacing such a defective gene with its functional equivalent. In particular, the WO 90/05180 application proposes, based on in vitro studies, to insert a functional RB¹¹⁰ gene into an RB-minus tumor
30 cell by means of a retroviral vector in order to render such cells non-malignant.

In addition, international application WO 89/06703 (by Dryja et al., having an international filing date of 23 January 1989, and published 27 July
35 1989) proposes the treatment of retinoblastoma defective tumors by administering a retinoblastoma gene expression product.

In this connection, it has been reported that the introduction of the RB¹¹⁰ gene into RB-minus retinoblastoma, osteosarcoma, bladder and prostate carcinoma cells resulted in cells showing reduced tumorigenicity in nude mice, but probably not a reduced cell growth rate. The results varied depending on the particular parental cell line (Goodrich et al., 1992, Cancer Research 52:1968-1973; Banerjee, A., et al., 1992, Cancer Research, 52:6297-6304; Takahashi, R., et al., 1991, Proc. Natl. Acad. Sci., USA, 88:5257-5261; Xu, H-J., et al., 1991, Cancer Research, 51:4481-4485; Bookstein et al, 1990, Science, 247:712-715; Huang, H-J.S., et al., 1988, Science 242, 1563-1566). However, the suppression of tumorigenicity by introduction of the p110^{RB} coding gene into RB-minus tumor cells is incomplete. The p110^{RB} reconstituted tumor cells still form invasive tumors in nude mice (Xu, H-J., et al., 1991, Cancer Research, 51:4481-4485; Takahashi, R., et al., 1991, Proc. Natl. Acad. Sci., USA, 88:5257-5261; Banerjee, A., et al., 1992, Cancer Research, 52:6297-6304). In particular, it has been shown that p110^{RB} reconstituted retinoblastoma cells inoculated into an orthotopic site (in this instance, the eye) consistently produced tumors (Xu, H-J., et al., 1991, Cancer Research 51:4481-4485). These findings, which will be discussed in detail *infra*, caution that the tumor suppressor gene replacement therapy as heretofore envisioned may simply result in cells that only appear to be "cured". Certainly, the findings of Xu et al. indicate a need for an improved genetic therapy for tumors which avoids these shortcomings.

Another proposed method of treating cancer by gene therapy is to antagonize the function of an oncogene by placing an artificial gene, constructed to have an inverted nucleotide sequence compared to the oncogene, into a tumor cell (U.S. patent number

4,740,463, issued April 26, 1988 by Weinberg, et al.).

All of these proposed solutions also share the deficiency of requiring that the specific genetic defect of the tumor to be treated be identified prior to treatment.

Since the p110^{RB} protein product is active in the underphosphorylated state (discussed in detail *supra*), and phosphoamino acid analysis has demonstrated only phosphoserine and phosphothreonine but not phosphotyrosine in RB protein (Shew, J-Y., et al., 1989, Oncogene Research, 1:205-213), it has been proposed to make a mutant RB protein with its serine or threonine residues being replaced by alanine or valine or others and that introduction of such a mutant, unphosphorylated RB protein into target cells may lead to growth arrest (International Application WO 91/15580, Research Development Foundation, by Fung et al., at page 20). Unfortunately, in all cases analyzed so far, the human RB protein carrying a point mutation and retaining the unphosphorylated state were invariably inactive proteins and associated with tumorigenesis rather than tumor suppression (Templeton et al., 1991, Proc. Natl. Acad. Sci., USA, 88:3033-3037).

25

1.6 Tumor Suppressor Gene Resistance

As the above discussion of gene mutations in tumor cells has indicated, not every cancer gene is a suitable candidate for wild-type gene replacement therapy due to the gene size or complexity or for other reasons. The retinoblastoma gene is one of those tumor suppressor genes that is readily accessible to study, thus it provides a model for understanding some of the other disadvantages to cancer gene replacement therapy as heretofore understood.

It is known that reintroduction of the retinoblastoma tumor suppressor gene into RB-defective tumor cells inhibits the tumor cell growth and suppresses the neoplastic phenotype of the target
5 cells (WO 90/05180, cited *supra*; Huang et al., 1988, Science, 242:1563-1566; Bookstein et al., 1990, Science, 247:712-715; Xu et al., 1991, Cancer Res., 51:4481-4485; Takahashi et al., 1991, Proc. Natl. Acad. Sci., USA, 88:5257-5261; Goodrich et al., 1992,
10 Cancer Res., 52:1968-1973; Banerjee et al., 1992, Cancer Res., 52:6297-6304).

However, the suppression of tumorigenicity is often incomplete. A significant percentage of the RB-reconstituted tumor cells still form small tumors
15 after a longer latency period in nude mouse tumorigenicity assays. Such tumors, although retaining normal RB expression, are histologically malignant and invasive (Xu et al., 1991, Cancer Res., 51:4481-4485; Takahashi et al., 1991, Proc. Natl. Acad. Sci., USA, 88:5257-5261; Banerjee et al., 1992,
20 Cancer Res., 52:6297-6304).

Furthermore, it has been observed that several cell lines derived from such RB-positive tumors have become very tumorigenic and have formed large,
25 progressively growing tumors when subsequently injected into nude mice (Zhou, Y.; Li, J.; Xu, K.; Hu, S-X.; Benedict, W.F., and Xu, H-J., Proc. Am. Assoc. Cancer Res., 34:3214, 1993). This phenomenon, which is referred to herein as tumor suppressor gene
30 resistance (TSGR) is a serious obstacle to the successful implementation of any scheme of tumor suppressor gene therapy for human cancers.

Without wishing to be bound by any particular hypothesis or explanation of the TSGR phenomenon, it
35 is believed that the RB gene product exemplifies a possible explanation for TSGR. RB proteins have an active form (underphosphorylated protein) and an

inactive form (phosphorylated protein). Therefore, RB-positive tumor cells may have inherited or acquired the ability to phosphorylate RB proteins to the inactive state and allow tumor cell proliferation to continue. Thus, conversion of RB-minus cells with plasmid or virus vectors coding for the p110^{RB} protein provides only incomplete suppression, or even exacerbation of a percentage of the malignant cell population because the p110^{RB} protein remains phosphorylated and inactive in some of the target cells.

Alternatively, the tumor cells expressing the RB¹¹⁰ gene may simply have again inactivated the RB¹¹⁰ gene by mutation in subsequent cell divisions (Lee et al., 1990, Immunol. Ser. 51:169-200, at page 188). Thus, there remains a need for a method of treating tumor cells by gene therapy so that the possibility of further mutation and resurgence of malignancy is avoided.

20

1.7 Summary of Obstacles to Cancer Gene Therapy

In brief, there are at least three major obstacles to be overcome to achieve a practical tumor suppressor gene therapy for tumor cells:

25 1) The necessity to determine the identity and sequence of each defective tumor suppressor gene or oncogene before attempting genetic therapy of that tumor. This is particularly a problem considering the multiple genetic defects found in many tumor cells studied;

30

2) The size and complexity of certain tumor suppressor genes or oncogenes renders manipulation of certain of these genes difficult; and

3) The possibility that TSGR as described above for the RB¹¹⁰ model system will generate tumor cells

35

that have equal or greater dysfunction than did the original abnormal cells.

Accordingly, there is a need in the art for a genetic therapy for tumor or cancer cells which can
5 safely overcome these problems and provide an effective treatment for all types of tumor cells without the need to determine the exact genetic deficiency of each treated tumor cell and without the risk of TSGR resurgence and exacerbation of the
10 malignancy.

2. SUMMARY OF THE INVENTION

Obstacles to the successful practice of tumor suppressor gene therapy of cancers are avoided by the
15 present invention. In a totally unexpected and surprising discovery, it has been determined that the second in-frame AUG codon-initiated retinoblastoma suppressor protein of about 94 kD (p94^{RB}) is a broad-spectrum tumor suppressor, and that insertion of a
20 gene capable of expressing this protein, or the protein itself, into an abnormally proliferating cell, such as a cancer or tumor cell, causes that cell to enter a senescent-like state, terminating the proliferation. The cell so-treated simply stops
25 replicating and dies. The cell may possess any type of genetic defect, known or unknown, so that there is no need to determine the exact nature of the genetic defect associated with the abnormal proliferation. Further, the population of treated cells exhibits an
30 unexpectedly much lower incidence of TSGR resurgence and exacerbation of malignancy than do cells treated with any other tumor suppressor gene. The method is repeated as needed.

Therefore, the invention provides p94^{RB} encoding
35 vectors and p94^{RB} proteins for use in treatment of tumors or cancers, and methods of preparing p94^{RB} proteins suitable for use in methods of treatment.

The invention also provides methods of treatment for mammals such as humans, as well as methods of treating abnormally proliferating cells, such as cancer or tumor cells. Broadly, the invention contemplates
5 treating abnormally proliferating cells, or mammals having a disease characterized by abnormally proliferating cells by any suitable method known to permit a host cell compatible p94^{RB} encoding vector or a p94^{RB} protein to enter the cells to be treated so that
10 suppression of proliferation is achieved.

~~In one embodiment, the invention comprises a~~
method of treating a disease characterized by abnormally proliferating cells, in a mammal, by administering an expression vector coding for p94^{RB} to
15 the mammal having a disease characterized by abnormally proliferating cells, inserting the expression vector into the abnormally proliferating cells, and expressing p94^{RB} in the abnormally proliferating cells in an amount effective to suppress
20 proliferation of those cells. The expression vector is inserted into the abnormally proliferating cells by viral infection or transduction, liposome-mediated transfection, polybrene-mediated transfection, CaPO4 mediated transfection and electroporation. The
25 treatment is repeated as needed.

In another embodiment, the invention comprises a method of treating abnormally proliferating cells of a mammal by inserting a p94^{RB} encoding expression vector into the abnormally proliferating cells and expressing
30 p94^{RB} therein in amounts effective to suppress proliferation of those cells. The treatment is repeated as needed.

In another alternative embodiment, the invention provides a DNA molecule able to suppress growth of an
35 abnormally proliferating cell. The DNA molecule encodes a p94^{RB} protein having an amino acid sequence

substantially according to SEQ ID NO:3, provided that the DNA molecule does not also code for a p110^{RB} protein. In a more preferred embodiment, the DNA molecule has the DNA sequence of SEQ ID NO:1, and is
5 expressed by an expression vector. The expression vector may be any host cell-compatible vector. The vector is preferably selected from the group consisting of a retroviral vector, an adenoviral vector and a herpesviral vector.

10 In another alternative embodiment, the invention provides a p94^{RB} protein having an amino acid sequence substantially according to SEQ ID NO:3.

In another alternative embodiment, the invention provides a method of producing a p94^{RB} protein by the
15 steps of: inserting a compatible expression vector comprising a p94^{RB} encoding gene into a host cell and causing the host cell to express p94^{RB} protein.

In another alternative embodiment, the invention comprises a method of treating abnormally
20 proliferating cells of a mammal ex vivo by the steps of: removing a tissue sample in need of treatment from a mammal, the tissue sample comprising abnormally proliferating cells; contacting the tissue sample in need of treatment with an effective dose of an p94^{RB}
25 encoding expression vector; expressing the p94^{RB} in the abnormally proliferating cells in amounts effective to suppress proliferation of the abnormally proliferating cells. The treatment is repeated as necessary; and the treated tissue sample is returned to the original
30 or another mammal. Preferably, the tissue treated ex vivo is blood or bone marrow tissue.

In another alternative embodiment, the invention comprises a method of treating a disease characterized by abnormal cellular proliferation in a mammal by a
35 process comprising the steps of administering p94^{RB} protein to a mammal having a disease characterized by

abnormally proliferating cells, such that the p94^{RB} protein is inserted into the abnormally proliferating cells in amounts effective to suppress abnormal proliferation of the cells. In a preferred embodiment, the p94^{RB} protein is liposome encapsulated for insertion into cells to be treated. The treatment is repeated as necessary.

In another alternative embodiment the invention comprises a method of treating abnormally proliferating cells of a mammal ex vivo by a process comprising the steps of removing a tissue sample in need of treatment from a mammal, the tissue sample comprising abnormally proliferating cells contacting the tissue sample in need of treatment with an effective dose of a p94^{RB} protein. The treatment is repeated as necessary, and then the treated tissue is returned to the mammal or placed into another mammal.

In a more preferred embodiment the tumor or cancer cells to be treated are cells having one or more genetically defective tumor suppressor genes and oncogenes selected from the group consisting of an RB, a p53, a c-myc, an N-ras and a c-yes-1 gene.

In a more preferred embodiment the tumor or cancer cells are cells having no detectable genetic defect of a tumor suppressor gene selected from the group consisting of an RB gene and a p53 gene.

In a still more preferred embodiment the tumor or cancer cells are lung carcinoma cells.

In a still more preferred embodiment the p94^{RB} encoding expression vector or the p94^{RB} protein are administered by means of aerosol delivery of liposome-encapsulated p94^{RB} encoding expression vector or p94^{RB} protein into a lung in need of such treatment.

3. DETAILED DESCRIPTION OF THE INVENTION

3.1 Definitions

The terms "cancer" or "tumor" are clinically descriptive terms which encompass a myriad of diseases characterized by cells that exhibit unchecked and abnormal cellular proliferation. The term "tumor", when applied to tissue, generally refers to any abnormal tissue growth, i.e., excessive and abnormal cellular proliferation. A tumor may be "benign" and unable to spread from its original focus, or "malignant" and capable of spreading beyond its anatomical site to other areas throughout the hostbody. The term "cancer" is an older term which is generally used to describe a malignant tumor or the disease state arising therefrom. Alternatively, the art refers to an abnormal growth as a neoplasm, and to a malignant abnormal growth as a malignant neoplasm.

Irrespective of whether the growth is classified as malignant or benign, the causes of excessive or abnormal cellular proliferation of tumor or cancer cells are not completely clear. Nevertheless, there is persuasive evidence that abnormal cellular proliferation is the result of a failure of one or more of the mechanisms controlling cell growth and division. It is also now believed that the mechanisms controlling cell growth and division include the genetic and tissue-mediated regulation of cell growth, mitosis and differentiation. These mechanisms are thought to act at the cell nucleus, the cell cytoplasm, the cell membrane and the tissue-specific environment of each cell. The process of transformation of a cell from a normal state to a condition of excessive or abnormal cellular proliferation is called tumorigenesis.

It has been observed that tumorigenesis is usually a multistep progression from a normal cellular state to, in some instances, a full malignancy. It is

therefore believed that multiple "hits" upon the cell regulatory mechanisms are required for full malignancy to develop. Thus, in most instances, it is believed that there is no single cause of excessive
5 proliferation, but that these disorders are the end result of a series of cumulative events.

While a malignant tumor or cancer capable of unchecked and rapid spread throughout the body is the most feared and usually the deadliest type of tumor,
10 even so-called benign tumors or growths can cause significant morbidity and mortality by their inappropriate growth. A benign tumor can cause significant damage and disfigurement by inappropriate growth in cosmetically sensitive areas, or by exerting
15 pressure on central or peripheral nervous tissue, blood vessels and other critical anatomical structures.

A broad-spectrum tumor suppressor gene is a genetic sequence coding for a protein that, when
20 inserted into and expressed in an abnormally proliferating host cell, e.g., a tumor cell, suppresses abnormal proliferation of that cell irrespective of the cause of the abnormal proliferation. The second in-frame AUG (ATG in DNA)
25 codon-initiated retinoblastoma gene disclosed herein exemplifies such a broad-spectrum tumor suppressor gene and is referred to herein as the p94^{RB} coding gene, as the RB⁹⁴ gene or as a DNA molecule coding for pRB⁹⁴. According to the nucleotide sequence of the
30 retinoblastoma susceptibility gene (McGee, T.L., et al., 1989, Gene, 80:119-128), the p94^{RB} coding gene comprises the nucleotide sequence from exon 3, nucleotide 355 to exon 27, nucleotide 264. Thus, the p94^{RB} encoding gene by definition excludes that portion
35 of the RB¹¹⁰ gene upstream from the second in-frame AUG start codon. Figures 1A-1F show the DNA sequence of

the RB⁹⁴ gene wherein the ATG codon begins at nucleotide 19 of that figure (SEQ ID NO:1; SEQ ID NO:2).

A broad-spectrum tumor suppressor protein
5 (including phosphoproteins, lipoproteins, glycoproteins and other protein-based derivatives) is a substance that when injected into, absorbed by or caused to be expressed in any abnormally proliferating cell, reduces or completely suppresses abnormal
10 cellular proliferation. The protein expressed by the second in-frame AUG codon-initiated retinoblastoma gene disclosed herein exemplifies such a broad-spectrum tumor suppressor protein. It is a phosphoprotein of about 94 kD relative molecular mass,
15 and is also referred to herein as p94^{RB} (SEQ ID NO:3).

One of ordinary skill in the art will be able to determine if any other fragment of a tumor suppressor protein, e.g., the third or fourth AUG codon-initiated retinoblastoma protein of about 90 kD and 83 kD,
20 respectively, also has the property of suppressing abnormal cellular proliferation.

3.2 Brief Description of the Figures

Figures 1A-1F: Nucleotide sequence of the cDNA
25 fragment encoding the 94 kD therapeutic RB protein (plus strand is SEQ ID NO:1, minus strand is SEQ ID NO:2).

Figures 2A-2F: Amino acid sequence of the 94 kDa therapeutic RB protein (SEQ ID NO:3).

30 Figure 3: Construction of baculovirus expression vector for the 94 kDa therapeutic RB protein synthesis; *R.S. is recombination sequence.

Figures 4A and 4B: Intracellular localization of recombinant baculovirus-produced p110^{RB} and p94^{RB} in
35 insect cells: Figure 4A shows mock-infected Sf9 cells; Figure 4B shows cells producing p110^{RB}; and Figure 4C shows cells producing p94^{RB}; note that

protein is localized to the nucleus in Figures 4B and 4C. Protein localization is by anti-RB immunochemical staining.

Figure 5: A diagram of complex formation of baculovirus-expressed and subsequently purified p110^{RB} and p94^{RB} proteins with SV40 T antigen. The immunoaffinity chromatography purified proteins were mixed with an equal amount of T antigen, and aliquots of the mixture were immunoprecipitated with PAB419 anti-T antibody, followed by Western blotting. The blot was sequentially incubated with MAb-1 anti-RB antibody and PAB419 antibody. Lane 1, lysate of T antigen immortalized W138 VA13 fibroblasts was used as a control; lane 2, purified p110^{RB}; lane 3, co-precipitation of T-Ag with p110^{RB}; lane 4, purified p94^{RB}; lane 5, co-precipitation of T-Ag with p94^{RB}.

Figures 6A and 6B: Construction of recombinant plasmids for high-level expression of p110^{RB} (pCMV-f-RB35) and p94^{RB} (pCMV-s-RB42) proteins in human cells using cytomegalovirus promoter/enhancer: Figure 6A is an explanatory drawing of the p110^{RB} coding cDNA; Figure 6B provides maps of the p110^{RB} and p94^{RB} expression plasmids where pCMV-f-RB35 codes for p110^{RB} and pCMV-s-RB42 codes for p94^{RB}. Note that pCMV-s-RB42 has most of p110^{RB} coding region deleted upstream of the second ATG.

Figures 7A and 7B: Construction of recombinant plasmids for expression of p110^{RB} (pBA-f-RB33) and p94^{RB} (pBA-s-RB34) proteins in human cells using β -actin promoter: Figure A is a map of the p110^{RB} coding plasmid, pBA-f-RB33; Figure B is a map of the p94^{RB} coding plasmid, pBA-s-RB34. Note that pBA-s-RB34 has most of the p110^{RB} coding region deleted upstream of the second ATG.

Figures 8A, 8B and 8C: Morphological effects of p110^{RB} and p94^{RB} expression on RB-defective bladder carcinoma cell line 5637 (ATCC HTB9): Figure 8A is

mock-transfected HTB9 cells; Figure 8B is p110^{RB} expressing HTB9 transfectants; Figure 8C is p94^{RB}-expressing HTB9 transfectants. Arrows indicate examples for RB-positive immunostained cells. Note
5 that the p110^{RB} expressing cells of Figure 8B appear normal, but that the p94^{RB} expressing cells of Figure 8C are senescent.

Figure 9. Half-life analysis of p110^{RB} and p94^{RB} proteins in RB-reconstituted bladder carcinoma cell
10 line, 5637. The bladder tumor cells were transfected in multiple dishes with either p110^{RB} (pBA-f-RB33) or p94^{RB} (pBA-s-RB34) expression plasmids. Twenty-four hours after transfection the cells were labeled with [³⁵S]-methionine and chased with excess unlabeled
15 methionine for 0, 6, 12 and 24 hours, respectively. The p110^{RB} and p94^{RB} proteins were determined by immunoprecipitation: the left side of the figure (0-12 hours) shows the half-life of p110^{RB} is less than 6 hours; the right side of the figure (0-24 hours) shows
20 the half-life of p94^{RB} is about 12 hours.

Figure 10. Western blot analysis of exogenous p110^{RB} and p94^{RB} proteins in transiently transfected 5637 cells showing the distinct underphosphorylation state of the p94^{RB} protein: lane 1 shows normal human
25 fibroblast cell line, WI-38; lane 2 shows parental RB-minus bladder carcinoma cell line, 5637; lane 3 shows 5637 cells transfected with p110^{RB}-expressing plasmid; lane 4 shows 5637 cells transfected with p94^{RB}-expressing plasmid.

30 Figures 11A-11C. Expression of the human full-length RB protein, p110^{RB} (Figure 11B) and the broad-spectrum tumor suppressor protein, p94^{RB} (Figure 11C) in normal (non-tumorigenic) mouse fibroblast cells via retrovirus plasmid vectors. Both the p110^{RB}- and p94^{RB}-
35 expressing cells (arrows) have normal viable morphology similar to the parental cells Figure 11A).

Figures 12A-12D. Expression of the human p110^{RB} (Figure 12C) and p94^{RB} (Figure 12D) proteins in mouse urinary bladder mucous membranes *in vivo*. Liposomes were mixed with the p110^{RB}- and p94^{RB}- expressing
5 plasmids, respectively and infused directly into the mouse bladder via a catheter. Notably, transitional epithelia expressing the p110^{RB} (Figure 12C, arrows) or p94^{RB} (Figure 12D, arrows) both retained normal, viable morphology similar to their counterparts in the
10 untreated mouse bladder (Figure 12A) or the mouse bladder treated with liposomes only (Figure 12B).

3.3 The Invention

The present invention is based upon the
15 unexpected discovery that p94^{RB} expressed by an expression vector in any abnormally proliferating target cell, e.g., a cancer or tumor cell, causes the suppression of the abnormal proliferation. Surprisingly, the treatment has been effective with
20 all tested tumor cell lines and is not limited to treatment of RB-minus tumor cells.

Without wishing to be bound by a particular hypothesis or proposed mechanism of action, it is believed that the p94^{RB} protein remains in the active,
25 underphosphorylated form, and has a half-life in the target cell which is two to three times longer than that of p110^{RB}. Thus, it is possible that a synergistic combination of accumulation of p94^{RB} together with its tendency to remain in an
30 underphosphorylated, active form serves to terminate the cell replication cycle in target tumor cells. However, whatever the mechanism of action, the property of suppressing cell growth and inducing senescence or killing any abnormally proliferating
35 cell, irrespective of its genetic defect, is nevertheless completely unanticipated and unexpected.

In order to obtain the broad-spectrum tumor suppressor protein, a gene coding for the second in-frame AUG codon-initiated RB protein, i.e., p94^{RB}, was expressed by a baculovirus vector in insect host cells as a stable nuclear phosphoprotein. The resulting unphosphorylated forms of p94^{RB} were able to form a specific complex with SV40 T antigen, providing an important verification that the p94^{RB} protein shares many functional properties of the naturally occurred p110^{RB} protein, i.e., phosphorylation, viral oncoprotein association and nuclear tethering (Templeton et al., 1991, Proc. Natl. Acad. Sci., USA, 88:3033-3037).

The effects of transfection by either first or second in-frame AUG codon-initiated RB protein expression plasmid were compared on a number of well known human tumor cell lines. The tested cell lines included: an RB-defective human bladder carcinoma cell line, 5637 (ATCC HTB9); RB-defective human breast carcinoma cell line, MDA-MB-468 (ATCC HTB132); RB-defective human non-small cell lung carcinoma cell line, H2009 (Kratzke, R.A., et al., 1992, The Journal of Biological Chemistry, 267:25998-26003); RB-defective human prostate carcinoma cell line, DU145 (ATCC HTB81); RB-defective human osteosarcoma cell line, Saos-2 (ATCC HTB85); RB-defective human fibrosarcoma metastatic to lung cell line, Hs913T (ATCC HTB152); human cervix adenocarcinoma cell line, HeLa (ATCC CCL2) and human fibrosarcoma cell line, HT1080 (ATCC CCL121). Both the HeLa and HT1080 cell lines have normal p110^{RB} expression. Each of these cell lines were separately transfected with the p110^{RB} coding and the p94^{RB} coding expression plasmids. The results demonstrated that the second in-frame AUG codon-initiated RB protein, p94^{RB}, was a more effective cell growth inhibitor, causing those dividing tumor

cells to senesce and die. On the other hand, most normal human cells in vivo are either non-dividing or have the potential to progress into the cell cycle after a long latency period. Therefore, p94^{RB}, as an
5 active cell cycle regulatory factor and a therapeutic reagent is expected to show little or no toxicity when transiently expressed in normal cells in vivo.

The study also demonstrated that the RB-minus tumor cells expressing the second in-frame AUG codon-
10 initiated RB protein, p94^{RB}, did not progress through the cell cycle, as evidenced by their failure to incorporate [³H]-thymidine into DNA. However, the percentage of cells undergoing DNA replication was only slightly lower in cells producing the intact RB
15 protein (p110^{RB}) than in cells that were RB-negative.

Of particular interest was the fact that the RB-defective bladder carcinoma cell line, 5637, failed to phosphorylate the second in-frame AUG codon-initiated RB protein as shown by Western blot analysis. In
20 contrast, the intact RB protein (p110^{RB}) expressed in transfected 5637 cells were fully phosphorylated. Moreover, the half-life of the second in-frame AUG codon-initiated RB protein, p94^{RB}, was shown to be two- to three-fold greater than the intact RB protein
25 (p110^{RB}). Therefore, the accumulation of only unphosphorylated (active) p94^{RB} proteins may account for the failure of transiently transfected 5637 tumor cells to enter S phase, and this in turn may cause these tumor cells to senesce and die.

30 In addition, it has also been found that p94^{RB} protein has its preferentially associated cellular proteins as compared to 100^{RB}. This difference in associated proteins may also contribute to the unique broad-spectrum tumor cell growth suppressing functions
35 of p94^{RB} protein.

Both the fibrosarcoma cell line, HT1080 and cervix carcinoma cell line, HeLa, which have normal RB gene expression, were also successfully treated with the second in-frame AUG codon-initiated RB protein (p94^{RB}) expression plasmid, demonstrating that expression of the p94^{RB} protein in RB+ cancer or tumor cells significantly suppressed the tumor cell growth. Therefore, an advantage of the present invention is that the methods and products herein disclosed can be used for therapeutic treating tumors having no specific tumor suppressor gene defects, which provides a significant advantage over previous techniques for human tumor suppressor gene therapy.

Table 1, on the following page, provides a summary of the identification of the tested tumor cell lines, their tumor origin and genetic defects.

20

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30

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TABLE 1: The Status of Antioncogenes (Tumor Suppressor Genes) and Oncogenes in p94^{RB}-Treated Human Tumor Cells

RECIPIENT CELLS	TUMOR ORIGIN	ANTIONCOGENES		ONCOGENES
		RB	p53	
5637	Bladder carcinoma, primary tumor	Negative	Mutation	
DU145	Prostate carcinoma, metastasis to brain	Point mutation	Mutation	
MDA-MB-468	Breast Carcinoma	Large deletion	Mutation	
H2009	Lung carcinoma	Mutation	Mutation	
Hs913T	Fibrosarcoma, metastasis to lung	Large deletion	Negative	
Saos2	Osteosarcoma, primary tumor	Large deletion	Negative	
HeLa	Cervix carcinoma, primary tumor	Normal	Negative	c-myc activation ¹
HT1080	Fibrosarcoma, primary tumor	Normal	Normal	N-ras and c-yes-1 activation ^{2,3}

¹ Durst, M., et al. Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. Proc. Natl. Acad. Sci., USA, 84(4):1070-1074, 1987.

² Brown, R., et al. A mechanism of activation of an N-ras gene in the human fibrosarcoma cell line HT1080. EMBO J., 3:1321-1326, 1984.

³ Sugawara, K., et al. Distribution of c-y s-1 gene product in various cells and tissues. Br. J. Cancer, 63(4):508-513, 1991.

3.3.1. Preparation of RB⁹⁴ Vectors

3.3.1.1. Therapeutic Vectors

Any of the methods known to the art for the
5 insertion of DNA fragments into a vector, as
described, for example, in Maniatis, T., Fritsch,
E.F., and Sambrook, J. (1989): Molecular Cloning (A
Laboratory Manual), Cold Spring Harbor Laboratory,
Cold Spring Harbor, New York; and Ausubel, F.M.,
10 Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G.,
Smith, J.A., and Struhl, K. (1992): Current Protocols
in Molecular Biology, John Wiley & Sons, New York, may
be used to construct p94^{RB} encoding gene expression
vectors consisting of appropriate
15 transcriptional/translational control signals and the
desired RB cDNA sequence downstream from the first in-
frame AUG codon, that is unable to code for p110^{RB}.
These methods may include *in vitro* DNA recombinant and
synthetic techniques and *in vivo* genetic
20 recombination. Expression of a nucleic acid sequence
encoding a p94^{RB} may be regulated by a second nucleic
acid sequence so that the p94^{RB} is expressed in a host
infected or transfected with the recombinant DNA
molecule. For example, expression of p94^{RB} may be
25 controlled by any promoter/enhancer element known in
the art. The promoter activation may be tissue
specific or inducible by a metabolic product or
administered substance.

Promoters/enhancers which may be used to control
30 p94^{RB} gene expression include, but are not limited to,
the native RB promoter, the cytomegalovirus (CMV)
promoter/enhancer (Karasuyama, H., et al., 1989, J.
Exp. Med., 169:13), the human β -actin promoter
(Gunning, P., et al., 1987, Proc. Natl. Acad. Sci.
35 USA, 84:4831-4835), the glucocorticoid-inducible
promoter present in the mouse mammary tumor virus long
terminal repeat (MMTV LTR) (Klessig, D.F., et al.,

1984, Mol. Cell Biol., 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss, R., et al., 1985, RNA Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early region promoter (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, Cell 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1444-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), the adenovirus promoter (Yamada et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82(11):3567-71), and the herpes simplex virus LAT promoter (Wolfe, J.H., et al., 1992, Nature Genetics, 1:379-384).

Expression vectors compatible with mammalian host cells for use in genetic therapy of tumor or cancer cells, include, but are not limited to: plasmids, retroviral vectors, adenovirus vectors, herpes viral vectors, and non-replicative avipox viruses, as disclosed, for example, by U.S. Patent No. 5,174,993.

In a specific embodiment, a plasmid vector derived from pH8APr-1-neo, was constructed for expression of p94^{RB} in mammalian cells by placing the coding sequence for p94^{RB} under control of the human β -actin gene promoter (Gunning, P. et al., Proc. Natl. Acad. Sci., USA, 1987, 84:4831-4835).

In another specific embodiment, a plasmid vector derived from pCMV-Neo-Bam (Baker, S.J., et al., Science, 1990, 249:912-915), was constructed for expression of p94^{RB} in mammalian cells by placing the coding sequence for p94^{RB} under control of the cytomegalovirus (CMV) promoter/enhancer sequences.

In another specific embodiment, a retroviral vector, pLLRNL (Miller, A.D., et al., 1985, Proc.

5 any other effective promoter.

10 to infect mammalian cells and express p94^{RB} protein under the control of the adenovirus type 2 (Ad2) major late promoter, the CMV promoter, the β -actin promoter or any other effective promoter.

15 3.3.1.2. Vectors for Production and
 Purification of p94^{RB}
 Protein

compatible host cells. These include but are not limited to mammalian cells infected with a virus (e.g., adenovirus, retrovirus, herpes simplex virus, avipox virus); insect cells infected with a virus (e.g., baculovirus); microorganisms such as yeasts containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression controlling elements of vectors vary in their strengths and specifications. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. The produced p94^{RB} may be purified from host cells by affinity chromatography, electrophoresis, high-performance liquid chromatography (HPLC) or any other methods known to the art.

35 In a specific embodiment an engineered derivative of Autographa californica Multiple Nuclear Polyhedrosis

Virus ("AcMNPV") was used to produce p94^{RB} protein in cultured Fall Army worm Spondoptera frugiperda cells (Sf9 cells) with a strong temporally regulated promoter of the polyhedron gene whose product
5 represents 50% or more of total cellular proteins during a lytic infection. The baculovirus-expressed p94^{RB} protein was subsequently purified by immunoaffinity chromatography.

10 3.3.1.3. Detection of p94^{RB} Coding
 Expression Vectors

Expression vectors containing p94^{RB} coding inserts can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of
15 "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a p94^{RB} coding gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that
20 are homologous/complementary to the inserted p94^{RB} coding gene. Such hybridization can be carried out under stringent or nonstringent conditions, depending upon the size and sequence of the probe selected. In the second approach, the expression vector/host system
25 can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, viral occlusion formation in a baculovirus vector infected insect cell, etc.) caused by introduction of
30 the expression vector into the host cell. For example, if the p94^{RB} coding gene is inserted within a vector having a dominant selectable marker gene, such as a neomycin phosphotransferase gene under separate control of an appropriate promoter, such as an SV40
35 early promoter, the expression vector containing the p94^{RB} coding gene can be identified by the presence of the marker gene function (geneticin resistance). In

the third approach, expression vectors containing a p94^{RB} coding gene can be identified by assaying the p94^{RB} coding gene products expressed by the vectors. Such assays can be based, for example, on the physical
5 or functional properties of the p94^{RB} gene products in *in vitro* or *in vivo* assay systems including metabolic radiolabelling by [³⁵S] methionine, SDS-polyacrylamide gel electrophoresis, binding with a specific antibody, and phosphorylation by a protein kinase.

10

3.3.2. Expression of p94^{RB}

An appropriate p94^{RB} coding expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the p94^{RB}
15 protein-coding sequence may be introduced into a host cell. A host cell may be any cell type compatible with the vector for expressing and producing p94^{RB}. In a preferred embodiment, the host cell is a mammalian tumor cell to be treated. In a more preferred
20 embodiment, the host cell is a human tumor cell to be treated. Expression of the p94^{RB} in a host cell may be transient, permanent, or inducible.

The necessary transcriptional and translational signals, including promoter/enhancer sequences can
25 also be supplied by the native RB gene and/or its flanking regions. A variety of vector/host systems may be utilized to express the p94^{RB} protein-coding sequence in a tumor cell to be treated. These include but are not limited to mammalian cell systems
30 transfected, infected or transduced with a plasmid, or a virus (e.g., adenovirus, retrovirus, herpes simplex virus, avipox virus). The expression elements of vectors vary in their strengths and specificities. Depending on the host cell to be treated, any one or
35 more of a number of suitable transcription and translation elements may be used.

3.3.3. Methods of Treatment

The p94^{RB} encoding gene construct of the present invention may be placed by methods well known to the art into an expression vector such as a plasmid or
5 viral expression vector. A plasmid expression vector may be introduced into a tumor cell by calcium phosphate transfection, liposome (for example, LIPOFECTIN)-mediated transfection, DEAE Dextran-mediated transfection, polybrene-mediated
10 transfection, electroporation and any other method of introducing DNA into a cell.

A viral expression vector may be introduced into a target cell in an expressible form by infection or transduction. Such a viral vector includes, but is
15 not limited to: a retrovirus, an adenovirus, a herpes virus and an avipox virus. When p94^{RB} is expressed in any abnormally proliferating cell, the cell replication cycle is arrested, thereby resulting in senescence and cell death and ultimately, reduction in
20 the mass of the abnormal tissue, i.e., the tumor or cancer. A vector able to introduce the gene construct into a target cell and able to express p94^{RB} therein in cell proliferation-suppressing amounts can be administered by any effective method.

25 For example, a physiologically appropriate solution containing an effective concentration of active vectors can be administered topically, intraocularly, parenterally, orally, intranasally, intravenously, intramuscularly, subcutaneously or by
30 any other effective means. In particular, the vector may be directly injected into a target cancer or tumor tissue by a needle in amounts effective to treat the tumor cells of the target tissue.

Alternatively, a cancer or tumor present in a
35 body cavity such as in the eye, gastrointestinal tract, genitourinary tract (e.g., the urinary bladder), pulmonary and bronchial system and the like

can receive a physiologically appropriate composition (e.g., a solution such as a saline or phosphate buffer, a suspension, or an emulsion, which is sterile except for the vector) containing an effective
5 concentration of active vectors via direct injection with a needle or via a catheter or other delivery tube placed into the cancer or tumor afflicted hollow organ. Any effective imaging device such as X-ray, sonogram, or fiberoptic visualization system may be
10 used to locate the target tissue and guide the needle or catheter tube.

In another alternative, a physiologically appropriate solution containing an effective concentration of active vectors can be administered
15 systemically into the blood circulation to treat a cancer or tumor which cannot be directly reached or anatomically isolated.

In yet another alternative, target tumor or cancer cells can be treated by introducing p94^{RB}
20 protein into the cells by any known method. For example, liposomes are artificial membrane vesicles that are available to deliver drugs, proteins and plasmid vectors both in vitro or in vivo (Mannino, R.J. et al., 1988, Biotechniques, 6:682-690) into
25 target cells (Newton, A.C. and Huestis, W.H., Biochemistry, 1988, 27:4655-4659; Tanswell, A.K. et al., 1990, Biochimica et Biophysica Acta, 1044:269-274; and Ceccoll, J. et al. Journal of Investigative Dermatology, 1989, 93:190-194). Thus, p94^{RB} protein
30 can be encapsulated at high efficiency with liposome vesicles and delivered into mammalian cells in vitro or in vivo.

Liposome-encapsulated p94^{RB} protein may be administered topically, intraocularly, parenterally,
35 intranasally, intratracheally, intrabronchially, intramuscularly, subcutaneously or by any other effective means at a dose efficacious to treat the

abnormally proliferating cells of the target tissue. The liposomes may be administered in any physiologically appropriate composition containing an effective concentration of encapsulated p94^{RB} protein.

5

3.3.4. Tumors Susceptible To Treatment

The gene construct and vectors of the present invention are effective in inhibiting the growth or mitosis or both of any type of tumor cell. The gene
10 construct of the invention has demonstrated effectiveness in treating tumor cells of carcinomas and sarcomas. In particular, the gene construct of the invention has demonstrated effectiveness in suppressing replication and inducing cell senescence
15 followed by cell death in the following tumor cell types: bladder carcinoma, lung carcinoma, breast carcinoma, prostate carcinoma, fibrosarcoma, osteosarcoma and cervix carcinoma.

Further, the gene construct of the invention has
20 demonstrated effectiveness in suppressing replication and inducing cell senescence followed by cell death in the tumor cells having the following identified genetic defects: tumor suppressor gene RB and p53 mutation, oncogene *myc* activation, and oncogene N-ras
25 and c-yes-1 activation.

Furthermore, the gene construct of the invention has demonstrated effectiveness in suppressing replication and inducing cell senescence followed by cell death in the tumor cells having normal endogenous
30 tumor suppressor RB¹¹⁰ and/or p53 gene expression.

In addition, the gene construct of the invention is able to suppress replication in lymphomas, leukemia and in tumor cells having tumor suppressor gene DCC and NF1 genetic defects, as well as in other tumor
35 cell types in which the genetic defects are unknown or have yet to be identified.

3.3.5. Ex Vivo Treatment of Tumor or Cancer Tissues

In a preferred embodiment a tumor cell is transduced with a retrovirus vector, an adenovirus
5 vector, a plasmid vector or any other appropriate vector capable of expressing the p94^{RB} protein in that tumor cell. The cancer cell may be present in a blood or bone marrow sample collected from a leukemia patient. A dose of p94^{RB} protein expressing retrovirus
10 vector or adenovirus vector or plasmid vector or any other appropriate vector is administered to the sample of blood or bone marrow at a dose sufficient to transduce enough cells in the sample to produce a reduction in tumor cell numbers. The cell
15 proliferation of the treated cancer cells will be slowed or terminated followed by a process similar to normal cellular differentiation or cell senescence. Analogously, blood or bone marrow or other tissue is treated ex vivo using an effective dose of a liposome-
20 encapsulated p94^{RB} protein. Thereafter the sample may be returned to the donor or infused into another recipient.

25 3.3.6. In Vivo Treatment of Tumor or Cancer Tissues

Methods of administering viral vectors are well known. In general, the skilled artisan will appreciate that a retroviral vector, an adenovirus
vector, a plasmid vector, or any other appropriate
30 vector capable of expressing the p94^{RB} protein can be administered in vivo to a cancer by a wide variety of manipulations. All such manipulations have in common the goal of placing the vector in sufficient contact with the target tumor to permit the vector to
35 transduce or transfect the tumor cells. In a preferred embodiment, cancers present in the epithelial linings of hollow organs may be treated by

infusing the vector suspension into a hollow fluid filled organ, or by spraying or misting into a hollow air filled organ. Thus, the tumor cell may be present in or among the epithelial tissue in the lining of pulmonary bronchial tree, the lining of the gastrointestinal tract, the lining of the female reproductive tract, genito-urinary tract, bladder, the gall bladder and any other organ tissue accessible to contact with the vector.

10 In another preferred embodiment, the cancer may be located in or on the lining of the central nervous system, such as, for example, the spinal cord, spinal roots or brain, so that vectors infused in the cerebrospinal fluid will contact and transduce the cells of the tumor in that space.

In another preferred embodiment, the cancer is a solid tumor. The skilled artisan will appreciate that the vector can be administered to the tumor by direct injection of the vector suspension into the tumor so that vectors will contact and transduce or transfect the tumor cells inside the tumor.

In yet another preferred embodiment, the cancer may be a cancer of the blood, blood forming organs or any organ directly perfused by the blood, so that vectors injected into the blood stream will contact and treat the cells of the cancer. Thus, the cancer may be a leukemia, a lymphoma or other tumor type and the tumor cell may be present in the blood, the bone marrow, the spleen, the thymus, the liver and any other blood perfused organ.

The skilled artisan will understand that the vector is administered in a composition comprising the vector together with a carrier or vehicle suitable for maintaining the transduction or transfection efficiency of the chosen vector and promoting a safe infusion. Such a carrier may be a pH balanced physiological buffer, such as a phosphate, citrate or

bicarbonate buffer, a saline solution, a slow release composition and any other substance useful for safely and effectively placing the vector in contact with abnormally proliferating cells to be treated.

5 The invention is further described in the following examples which are in no way intended to limit the scope of the invention.

4. Examples

10 4.1 Preparation of Vectors for Expression of the Second In-Frame AUG Codon-Initiated RB Protein in Insect Cells

 The engineered derivatives of Autographa californica Multiple Nuclear Polyhedrosis Virus ("AcMNPV") have been widely employed to produce high
15 levels of accurately processed and biologically active proteins. This baculovirus propagates in cultured Fall Army worm Spondoptera frugiperda cells (Sf9 cells) and has a strong temporarily regulated promoter of the polyhedron gene whose product represents 50% or
20 more of total cellular proteins during a lytic infection.

 By in vivo recombination, the coding sequence of a foreign gene can easily be placed under the transcriptional control of the polyhedron promoter,
25 resulting in a high level of expression. In addition, such proteins may be correctly folded and contain appropriate post-translational modifications like those proteins in the native higher eukaryotes.

 By site-specific mutagenesis, two BamH1 sites
30 were introduced into the RB cDNA at nucleotides +7 and +3230 (the A of the second in-frame AUG codon is designated +19). The resulted DNA molecule has the nucleotide sequence of Figure 1 (SEQ ID NO:1; SEQ ID NO:2), which is also referred to herein as the second
35 in-frame AUG codon-initiated RB protein gene, or the p94^{RB} encoding gene. The coded-for protein has the

sequence of Figure 2 (SEQ ID NO:3) and is referred to herein as the second in-frame AUG codon-initiated RB protein, or the p94^{RB} protein.

In an attempt to achieve maximal production of the second in-frame AUG codon-initiated RB protein in the baculovirus expression system, the recombinant transfer vector was constructed with insertion of the p94^{RB} gene into the pVL1393 plasmid so that the p94^{RB} gene was placed under the control of the polyhedron gene promoter.

As shown in Figure 3, the resulting pVL-s-RB plasmid contains no additional AUG start codon upstream from the p94^{RB} translation initiation site at nucleotide +19, and thus encodes a nonfusion p94^{RB} protein. In a parallel study, the same strategy was employed to construct a p110^{RB} expression vector which was designated pVL/1st AUG-RB.

Transfer of RB cDNAs from the recombinant vectors to the viral genome was accomplished by co-transfecting wild-type AcMNPV virus DNA with pVL-s-RB plasmid DNA or pVL/1st AUG-RB plasmid DNA. The recombinant viruses were subjected to three rounds of plaque purification to obtain a pure stock of RB-containing baculovirus, designated AcMNPV-RB94 and AcMNPV-RB110, respectively.

4.2 Purification of p110^{RB} and p94^{RB} Proteins

The p110^{RB} and p94^{RB} proteins were purified from baculovirus-infected insect cells by immunoaffinity chromatography. Briefly, insect cells were harvested 24 hours after the virus infection and lysed at 4°C with EBC buffer (50 mM Tris-HCl, pH8.0, 120 mM NaCl, 0.5% NP-40, 50µg/ml aprotinin). The lysate was clarified by centrifugation and the p110^{RB}- or p94^{RB}-containing supernatant was incubated with biotinylated WL-1 polyclonal anti-RB antibodies (Xu, H-J., et al., 1989, Oncogene, 4:807-812) at 4°C overnight. The

procedures for biotinylation of rabbit IgGs using succinimide ester followed the methods described by Bayer and Wilchek (Baylor, E.A. and Wilchek, M., 1980, Methods Biochem. Anal., 26:1-45). The RB protein-IgG-
5 biotin complex was collected on a streptavidin agarose gel column. Purified p110^{RB} or p94^{RB} were eluted from separate columns using 100 mM glycine (pH 2.2) and neutralized with 1M of phosphate (pH 8.0).

10 4.2.1. p94^{RB} Shares Major Biochemical and Biological Properties With p110^{RB}

Since non-functional mutations of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association and
15 nuclear localization (Templeton et al., 1991, Proc. Natl. Acad. Sci., USA, 88:3033-3037), the functional aspects of the artificial p94^{RB} protein were studied for these characteristics.

First, to determine whether the RB proteins
20 produced in the insect cells with the baculoviruses were associated with the nucleus, the AcMNPV-RB110 and AcMNPV-RB94 infected Sf9 cells were immunostained with MAb-1 anti-RB monoclonal antibody 24h after infection. As shown in Figure 4, intense staining was found
25 exclusively in the nuclei of cells infected with either AcMNPV-RB110 (panel B) or AcMNPV-RB94 (panel C).

The p110^{RB} and p94^{RB} proteins purified from baculovirus-infected insect cells by immunoaffinity
30 chromatography were tested for their ability to form a specific complex with SV40 T antigen. Briefly, equal amounts of p94^{RB} or p110^{RB} and T antigen were mixed and aliquots of the mixture were immunoprecipitated with PAB419 anti-T antibody. As shown in Figure 5, mixing
35 of p94^{RB} (or p110^{RB}) with T antigen in vitro resulted in the co-immunoprecipitation of both under- and hypo-phosphorylated p94^{RB} (lane 5), or p110^{RB} (lane 3) with

PAB419. The data demonstrated that either p110^{RB} or p94^{RB} protein can form a specific complex with SV40 T antigen. The AcMNPV-RB94 virus-infected insect cells appear to make hyperphosphorylated p94^{RB} (lane 4),
 5 which was unable to form complexes with SV40 T antigen (compare lane 4 with lane 5).

The Western blot shown in Figure 5 revealed an apparent relative molecular mass (Mr) of 94 kD for the second in-frame AUG codon-initiated RB protein. On
 10 SDS-PAGE, the p94^{RB} protein (Figure 5, lanes 4 and 5) was smaller than the naturally occurring 98 kDa proteins of unknown origin (Xu et al., 1989, Oncogene, 4:807-812) (Figure 5, lane 1). Therefore, the second in-frame AUG codon-initiated RB protein of this
 15 invention (p94^{RB}) has not been found to occur naturally in human cells.

It is concluded that the second in-frame AUG codon-initiated p94^{RB} protein produced in recombinant virus-infected insect cells is a artificial but stable
 20 nuclear phosphoprotein with its under- and hypo-phosphorylated forms being able to assemble specific complex with SV40 T antigen, as does the naturally occurring RB protein species, p110^{RB}.

25 4.3 Construction of Expression Vectors for Mammalian Cells

4.3.1. Subcloning of RB cDNA Fragments Encoding for the First and Second In-Frame AUG Codon-Initiated RB Proteins

30 Subcloning of RB cDNA fragments encoding for the first and second in-frame AUG codon-Initiated RB proteins was accomplished by standard methods in the art. The methods for DNA manipulation were modified from Maniatis, T., Fritsch, E.F., and Sambrook, J.
 35 (1989): Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; and Ausubel, F.M., Brent, R., Kingston, R.E.,

Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1992): Current Protocols in Molecular Biology, John Wiley & Sons, New York.

5 4.3.2. Preparation of a DNA Molecule
 Encoding the Second In-Frame AUG
 Codon-Initiated RB Protein.

 A plasmid, p4.95BT (Friend et al., 1987, Proc.
 Natl. Acad. Sci. USA, 84:9059-9063) or F7 (Takahashi,
10 R., Hashimoto, T., Xu, H-J., et al., 1991, Proc. Natl.
 Acad. Sci. USA, 88:5257-5261) containing the full
 length retinoblastoma (RB) gene cDNA was digested with
 the restriction enzyme, Hind II, at nucleotide +7 and
 the restriction enzyme, ScaI, at nucleotide 3,230
15 (The A of the second in-frame AUG codon of the full
 length RB cDNA open reading frame was designated
 nucleotide +19). The resulted 3,230 bp RB cDNA
 fragment had two blunt ends. Conversion of the blunt
 ends to restriction enzyme BamHI sites was done by
20 ligation of a synthetic BamHI oligonucleotide linker
 (GGGATCCC) to each blunt end of the fragment followed
 by digestion with the BamHI enzyme.

 The desired RB cDNA fragment was inserted into
 the BamHI cloning site of a plasmid vector, pUC19, and
25 propagated in the Escherichia coli strain, DH5 alpha
 bacterial cells. The recombinant plasmid was purified
 from a single DH5 alpha transformant and designated
 plasmid pUC-s-RB. This plasmid contains the desired
 RB cDNA fragment of 3,230 bp coding for the second
30 in-frame AUG codon-initiated RB protein of 816 amino
 acids.

 4.3.3. Preparation of A DNA Molecule
 Encoding the First In-Frame AUG
 Codon-Initiated RB Protein.

35 The full length RB cDNA plasmid was digested with
 the restriction enzyme, AcyI at nucleotide -322 and
 ScaI at nucleotide 3,230. The AcyI ends (overhang

5'-CG) were repaired by "filling in" the ends with the Klenow fragment of *E. coli* DNA polymerase I in the presence of all 4 dNTPs to generate blunt ends. Conversion of the blunt ends to restriction enzyme BamHI sites was done as described above. The resulted RB cDNA fragment of 3,552 bp was inserted into the plasmid pUC19 and propagated in the Escherichia coli strain DH5 alpha, which was subsequently purified from a single DH5 alpha transformant and designated plasmid pUC-f-RB. This plasmid contains the RB cDNA fragment of 3,552 bp coding for the first in-frame AUG codon-initiated RB protein of 928 amino acids.

4.3.4. Construction of p94^{RB} Expression Plasmid Using A Human β -Actin Gene Promoter

The RB cDNA fragment of 3,230 bp coding for the second in-frame AUG codon-initiated RB protein of 816 amino acids (p94^{RB}) was recovered from plasmid pUC-s-RB following the restriction enzyme, BamHI digestion, and re-inserted into the unique BamHI site of an expression plasmid, pHBApr-1-neo (Gunning, P., et al., Proc. Natl. Acad. Sci., USA, 1987, 84:4831-4835) in a orientation that the p94^{RB} coding sequence was under the direct control of the β -actin gene promoter. A plasmid vector with the correct insert orientation was selected by restriction endonuclease mapping after propagation in DH5 alpha Escherichia coli host cells, and was designated p β A-s-RB34 (Figure 7B). The corresponding DH5 alpha strain that contains plasmids p β A-s-RB34 was thereafter designated DHB-s-RB34 (ATCC 69241, patent depository, American Type culture Collection).

The plasmid vector p β A-s-RB34 contains no additional AUG codon between the β -actin gene promoter and the second in-frame AUG codon of the RB coding sequence, and thus encodes a non-fusion p94^{RB} protein.

The plasmid vector p β A-s-RB34 also confers a dominant selectable marker (geneticin resistance) in eukaryotic cells through expression of the neomycin phosphotransferase (neo) under separate control of an SV40 early promoter (Figure 7, sv-neo).

4.3.5. Construction of p110^{RB} Expression Plasmid Using A Human β -Actin Gene Promoter

10

The RB cDNA fragment of 3,552 bp coding for the first in-frame AUG codon-initiated RB protein of 928 amino acids (p110^{RB}) was recovered from plasmid pUC-f-RB and re-inserted into the expression plasmid pHBAPr-1-neo downstream from the β -actin gene promoter. The resulting plasmid vector was designated p β A-f-RB33 (Figure 7A). The plasmid vector p β A-f-RB33 contains no additional AUG codon between the β -actin gene promoter and the first in-frame AUG codon of the RB coding sequence, and thus encodes a non-fusion p110^{RB} protein.

4.3.6. Construction of p94^{RB} and p110^{RB} Expression Plasmids Using A Cytomegalovirus Promoter (CMVp)

25

Alternatively, an expression plasmid, pCMV-Neo-Bam (Baker, S.J., et al., Science, 1990, 249:912-915) was used in place of plasmid pHBAPr-1-neo. The vector included cytomegalovirus (CMV) promoter/enhancer sequences, which could drive expression of the insert at the BamHI site, and splicing and polyadenylation sites derived from the rabbit β -globin gene, which ensured proper processing of the transcribed insert in the cells. A pBR322 origin of replication and β -lactamase gene facilitated propagation of the plasmid in *E. coli*. The plasmid conferred geneticin resistance (a selectable marker in eukaryotic cells)

35

through expression of the neomycin phosphotransferase (neo) under the control of a herpes simplex virus (HSV) thymidine kinase promoter.

The same strategies as described supra in
5 Sections 4.3.4 and 4.3.5 were employed to transfer the
RB cDNA fragments of 3,230 bp and 3,552 bp from
plasmids pUC-s-RB and pUC-f-RB, respectively, to the
unique BamHI site in the expression vector, pCMV-Neo-
Bam. The resulting plasmid vectors were designated by
10 the names of pCMV-s-RB42, expressing the p94^{RB} and
pCMV-f-RB35, expressing the p110^{RB} (Figure 6). The
corresponding Escherichia coli DH5 alpha strain which
contains plasmids pCMV-s-RB42 was thereafter
designated DHC-s-RB42 (ATCC 69240, patent depository,
15 American Type Culture Collection).

4.3.7. Construction of p94^{RB} Protein Expression Retrovirus Vectors

For this protocol, retroviral vector, pLLRNL
20 (Miller, A.D., Law, M.-F., Verma, I.M., Molec. Cell
Biol., 1985, 5:431) and amphotropic retrovirus
packaging cell line, PA317 (ATCC CRL9078) (Miller,
A.D., and Buttimore, C., Molec. Cell Biol., 1986,
6:2895-2902) are used.

25 A plasmid p4.95BT or F7 containing the
full-length RB gene cDNA is digested with the
restriction enzyme Hind II at nucleotide +7 (the A of
the second in-frame AUG codon of the full-length RB
cDNA open reading frame was designated nucleotide
30 +19). Conversion of the Hind II site to restriction
enzyme Hind III site is done by ligation of a
synthetic Hind III oligonucleotide linker (CCAAGCTTGG)
to the blunt ends of the linear plasmid DNA, followed
by digestion with the Hind III enzyme. The linear
35 plasmid DNA is further digested with restriction
enzyme, ScaI, at nucleotide 3,230. The resulted RB
cDNA fragment of 3,230 bp codes for the second

in-frame AUG codon-initiated RB protein of 816 amino acids (p94^{RB}). This fragment has a 5'-Hind III site (cohesive end) and a 3'-ScaI site (blunt end), which facilitates its insertion into the retroviral vector, pLLRNL.

The vector pLLRNL is digested with two sets of restriction enzymes: Hind III/ClaI and SmaI/ClaI to delete the luciferase gene. Appropriate fragments are recovered from the agarose gel following electrophoresis, and ligated with the RB cDNA fragment of 3,230 bp to form a new vector, pLRB94RNL, in which the p94^{RB} expression is under the control of the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTRs).

The basic protocol for construction of the retroviral vector, pLRB94RNL, is modified from Huang, H.-J.S., et al., 1988, Science, 242:1563-1566.

Alternatively, the vector pLLRNL is digested with a single restriction enzyme, Hind III, to delete the luciferase gene, as well as the Rous sarcoma virus promoter (RSV). An appropriate DNA fragment is recovered from the p94^{RB} expression plasmid, pCMV-s-RB42 (or pBA-s-RB34). The recovered DNA fragment, which contains the 3,230 bp RB cDNA fragment and 5'-flanking CMV promoter (or β -actin promoter), is inserted into the ClaI restriction site of the retroviral vector. Conversion between the restriction enzyme sites is done by the methods as described *supra* in Section 4.3.7.

In the resulting p94^{RB} expression retrovirus vector, the p94^{RB} gene is under the control of an internal promoter (the CMV promoter or β -actin promoter), while the Tn5 neomycin-resistance gene (Neo) is under the control of the MuLV LTRs.

A safe and efficient amphotropic packaging cell line is necessary for transfer of retroviral vector genes into human cancer cells. The virus packaging

methods are modified from the method of Miyanohara et al., Proc. Natl. Acad. Sci., USA, 1988, 85:6538-6542. For this protocol, the PA317 packaging cell line is used. This packaging cell line has received prior
5 approval for use in human gene therapy clinical trials.

The retroviral vector (pLRB94RNL) DNA is transfected into PA317 packaging cells by LIPOFECTIN reagent (GIBCO BRL Life Technologies, Inc.,
10 Gaithersburg, MD) or electroporation methods as described in Sections 4.4.1. *infra*. Single colonies are isolated by selection in G418-containing medium (400 µg/ml) and expanded into mass cultures. To titer the virus produced by selected PA317 clones, dilutions
15 of cell-free culture medium from each PA317 clone are applied to 208F rat fibroblasts (indicator cells) in the presence of POLYBRENE (Sigma, 4 µg/ml) and G418 selection (400 µg/ml) is started 24 hours after infection.

20 After two weeks, G418-resistant colonies are visualized by Giemsa staining and viral titers are determined (colony-forming units per milliliter, cfu/ml). PA317 clones producing high virus titers are then assayed for human p94^{RB} protein expression by
25 Western immunoblotting as described previously (Xu, H.-J., et al., Oncogene, 1991, 6:1139-1146). Cell-free culture media from selected PA317 clones expressing high level of human p94^{RB} protein are then applied to human cancer cells ex vivo or in vivo.

30

4.3.8. Construction of p94^{RB} Protein Expression Adenovirus Vectors

The recombinant adenovirus Ad-RB94 is constructed from the adenovirus type 5 (Ad5) deletion mutant, Ad-dl324, and a plasmid, pTG5955, in which the human
35 CFTR cDNA has been replaced by the human RB cDNA fragment of 3,230 bp coding for the p94^{RB} protein. The

plasmid pTG5955 containing the RB cDNA insert is linearized by restriction enzyme ClaI cleavage and co-transfected with the large fragment of ClaI-cut Ad-dl324 DNA into 293 (ATCC CRL1573) cells to allow homologous recombination to occur, followed by replication and encapsidation of recombinant adenoviral DNA into infectious virions and the formation of plaques. Individual plaques are isolated and amplified in 293 cells, viral DNA is isolated and recombinant adenovirus plaques containing the human RB cDNA (Ad-RB94) are identified by restriction cleavage and Southern analysis. Ad-RB94 viruses are propagated in 293 cells and recovered 36 hours after infection. The viral preparation is purified by CsCl density centrifugation, and stored in virus dialysis buffer (10 mM Tris-HCl, pH7.4; 1 mM MgCl₂) at 4°C for immediate use; or stored at -70°C prior to use (with the addition of 10% glycerol). The basic protocol for construction of the recombinant adenovirus Ad-RB94 is modified from Rosenfeld, M.A., et al., Cell, 1992, 68:143-155.

4.3.9. Physical DNA Transfer Method

An alternative gene transfer method that has been approved for use in humans by the Food and Drug Administration is the transfer of plasmid DNA in liposomes directly to tumor cells in situ (Nabel, E.G., et al., 1990, Science, 249:1285-1288). Plasmid DNA is easy to certify for use in humans because, unlike retroviral vector, it can be purified to homogeneity.

The p94^{RB} expressing plasmid vectors pCMV-s-RB42 or pBA-s-RB34 are used to form complexes with liposomes, and directly treat tumor cells in vivo (or ex vivo). In this procedure, as described in Section 4.4.1 *infra*, stable integration of the DNA

into transfected tumor cells is not required for gene therapy as transient expression may suffice.

5 4.4. Treatment of Human Tumor Cells In Vitro With p94^{RB} Plasmid Vectors pBA-s-RB34 or pCMV-s-RB42.

4.4.1. Treatment of RB-Defective Human Tumor Cells In Vitro

Human tumor cells having known RB gene
10 deficiencies were treated with the p94^{RB} plasmid vector pBA-s-RB34 (or pCMV-s-RB42). These include: 1) human bladder carcinoma cell line, 5637, (ATCC HTB9); 2) human breast carcinoma cell line, MDA-MB-468 (ATCC HTB132); 3) human non-small cell lung carcinoma cell
15 line, H2009 (Kratzke, R.A., et al., 1992, The Journal of Biological Chemistry, 267:25998-26003); 4) human prostate carcinoma cell line, DU145 (ATCC HTB81); 5) human osteosarcoma cell line, Saos2 (ATCC HTB85); and 6) human fibrosarcoma metastatic to lung cell line,
20 Hs913T (ATCC HTB152).

For treatment, tumor cells were transiently transfected with the plasmid DNA pBA-s-RB34 (or pCMV-s-RB42) via LIPOFECTIN reagent (GIBCO BRL Life Technologies, Inc. Gaithersburg, MD). Similar results
25 have been obtained from transfection using calcium phosphate or electroporation methods.

The following procedures for transfection using LIPOFECTIN were modified from the manufacturer's specifications. Tumor cells were seeded in 100-mm
30 dishes in appropriate growth medium supplemented with serum. The cells were incubated at 37°C in a 5% CO₂ environment until the cells were 40-60% confluent. This usually took 18-24 hours, but the time varied among cell types. The following solution was prepared
35 in 17 x 75 mm polystyrene tubes: Solution A - for each dish of cells to be transfected, 5-10µg of plasmid DNA were diluted into a final volume of 100µl

with serum-free medium; Solution B - for each dish of cells to be transfected, 30-50 μ l of LIPOFECTIN reagent was diluted into a final volume of 100 μ l with serum-free medium. The two solutions were combined, mixed
5 gently, and incubated at room temperature for 10-15 min. The LIPOFECTIN reagent interacted spontaneously with plasmid DNA to form a lipid-DNA complex. While the lipid-DNA complex was forming, the cells were washed twice with 6 ml of serum-free medium. For each
10 transfection, 6 ml of serum-free medium were added to each polystyrene tube containing the lipid-DNA complex. The solution was mixed gently, and the medium-complex was overlayed onto the cells. The dishes were then swirled gently to ensure uniform
15 distribution. The dishes were then incubated at 37°C in a 5% CO₂ incubator. After 12 to 24 hours the medium-complex was replaced with appropriate growth medium containing 10% fetal calf serum.

In parallel studies, tumor cells were transfected
20 with the plasmid DNA pBA-f-RB33 or pCMV-f-RB35 which expresses the p110^{RB}. The following assays were used to evaluate the growth inhibitory effects of introducing p94^{RB} versus p110^{RB} expression in RB defective tumor cells:

25

- 1) DNA synthesis in tumor cells treated with plasmid vectors.

After plasmid DNA treatment the tumor cells were labeled with [³H]-thymidine for 2 hours, then
30 transferred to polylysine-coated slides, fixed and immunocytochemically stained with a monoclonal anti-RB antibody, MAb-1 (Triton Biosciences, Inc. Alameda, CA). The RB-positive transfected cells were counted under the microscope. The slides were then coated
35 with Kodak NTB2 autoradiographic emulsion and exposed for 7-10 days. The [³H]-thymidine labeling and RB protein immunocytochemical staining were done

according to the methods previously described (Xu et al., Oncogene, 1991, 6:1139-1146). About 400 to 1600 RB-positive and 600 RB-negative tumor cells were assessed for each determination of [³H]-thymidine uptake. The study demonstrated that the RB-defective tumor cells expressing p94^{RB} did not progress through the cell cycle, as evidenced by their failure to incorporate [³H]-thymidine into DNA (Table 2). However, the percentage of cells undergoing DNA replication was only slightly lower in cells producing p110^{RB} than in cells that were RB-negative (Table 2).

Table 2. Immunocytochemical Staining and [³H] Thymidine Incorporation of RB-Defective Tumor Cells Following Transfection With p94^{RB} or p110^{RB} Expression Plasmids

Recipient Cells	Promoter	Protein Expressed	Cells Incorporating RB+	[³ H] Thymidine RB-
5637	β -actin gene promoter	p110 ^{RB}	34%	45%
		p94 ^{RB}	2.3%	43%
	Cytomegalovirus promoter/enhancer	p110 ^{RB}	21%	—
		p94 ^{RB}	1.8%	---
MDA-MB-468	Cytomegalovirus promoter/enhancer	p110 ^{RB}	14%	40%
		p94 ^{RB}	0.5%	39%
H2009	β -actin gene promoter	p110 ^{RB}	19%	26%
		p94 ^{RB}	0.1%	27%
DU145	Cytomegalovirus Promoter/enhancer	p110 ^{RB}	23%	33%
		p94 ^{RB}	1.0%	33%
Hs913T	Cytomegalovirus promoter/enhancer	p110 ^{RB}	18%	34%
		p94 ^{RB}	0.9%	36%
Saos2	Cytomegalovirus promoter/enhancer	p110 ^{RB}	19%	32%
		p94 ^{RB}	0.9%	35%

2) Colony formation assay.

Approximately 48 hours after transfection the tumor cells were replated at a density of 10⁵ cells per 100 mm dish with selected medium containing G418 of 400-600 μ g/ml. Cells were cultured for 2 to 3 weeks

and colonies of >100 cells were scored. The data are illustrated in Table 3. Cells treated with plasmid vectors expressing p94^{RB} formed approximately four-fold fewer colonies than those transfected with p110^{RB} plasmid vectors. The difference was statistically significant (p <0.05 by t-test).

Furthermore, in those colonies that did form after p94^{RB} plasmid DNA treatment, p94^{RB} protein expression was no longer observed. Failure to isolate long-term cultures expressing the p94^{RB} protein in treated tumor cells shows that p94^{RB} did suppress tumor cell growth. In contrast, 7 of 48 cell lines (approximately 15%) derived from tumor cells after transfection with the p110^{RB} plasmid DNA were found to express the p110^{RB} protein. This percentage was consistent with results expected in human cells transfected with a vector containing two independent transcription units and therefore introduction of p110^{RB} expression does not exert growth inhibitory effects on RB-defective tumor cells.

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Table 3: Growth inhibitory effects of introducing p110^{RB} and p94^{RB} expression into RB-defective bladder carcinoma cell line, 5637 (HTB9). Each number represents 6 to 11 dishes.

5

	Vector Type	Number of G418-Resistant Colonies Formed		
		Vector	p110 ^{RB}	p94 ^{RB}
10	pCMV-Neo-Bam	280	24	6
	pHBAPr-I-neo	—	33	8

15 3) The Effect of p94^{RB} Expression on Cellular Morphology

The HTB9 transfectants were also immunostained with MAb-1 anti-RB monoclonal antibody about 24 hours after transfection. The staining results are
20 illustrated in Figure 8.

As demonstrated in Figure 8, the majority of RB-positive, p94^{RB}-expressing HTB9 cells become very large in size, with lower nucleocytoplasmic ratio, or higher incidence of being multinucleated cells
25 (panel C), a morphological change frequently associated with cellular senescence. However, such a morphological change has not been seen in group A, mock-transfected HTB9 cells and group B, p110^{RB} expressing RB-positive HTB9 cells (Figure 8, panels A
30 and B).

4.4.2. Treatment of Human Tumor Cells
Having Normal (p110^{RB}) RB
Expression (RB+)

Two RB+ human cell lines (i.e., having no RB gene
5 defect), including a human fibrosarcoma cell line,
HT1080 (ATCC CCL121), and human cervix carcinoma cell
line, HeLa (ATCC CCL2) were treated with the p94^{RB}
protein expression plasmid, pCMV-s-RB42, using the
LIPOFECTIN reagent as described *supra*. In parallel
10 studies, these cell lines were also transfected with
the p110^{RB} protein expression plasmid, pCMV-f-RB35.
The colony formation assay as described *supra* was used
to evaluate the growth inhibitory effects of
introducing exogenous p94^{RB} versus p110^{RB} expression in
15 RB+ tumor cells. As shown in Table 4, expression of
the p94^{RB} protein dramatically inhibited the cell
growth of HT1080 and HeLa cells. There was a two- to
nine-fold reduction in the number of G418-resistant
colonies formed after treated with the plasmid vector
20 pCMV-s-RB42 expressing p94^{RB}, while no such effect was
observed by transfection with the pCMV-f-RB35 plasmid
(expressing p110^{RB} protein). The difference was
statistically significant (the two-tailed P values
were less than 0.03 as calculated by the paired t-
25 test).

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Table 4: Growth inhibitory effects of introducing p110^{RB} and p94^{RB} expression into RB-positive human fibrosarcoma cell line, HT1080 and the RB positive human cervix carcinoma cell line, HeLa. The RB expression was under the control of cytomegalovirus (CMV) promoter. For each experiment, three 5-cm² dishes were transfected and the total colonies counted after ten days of selection in G418 (1 mg/ml).

No. of G418-Resistant Colonies Formed				
Recipient Cells	Experiment	Vector	p110 ^{RB}	p94 ^{RB}
HT1080	1	94	129	14
	2	88	122	16
	3	100	120	17
	4	99	110	15
HeLa	1	24	20	10
	2	25	24	9

4.5 Half-Life and Phosphorylation state of the p94^{RB} Protein In Host Cells: The Distinct Properties of p94^{RB}

The half-life of transiently expressed p94^{RB} and p110^{RB} proteins in transfected bladder carcinoma cell line, 5637 (ATCC HTB9) was measured by pulse-labeling of transfected 5637 cells with [³⁵S]-methionine followed by a chase with excess unlabeled methionine (Figure 9).

The bladder tumor cells were transfected in multiple dishes with either p110^{RB} (Figure 9, left) or p94^{RB} (Figure 9, right) expression plasmids. Twenty-four hours after transfection the cells were labeled with [³⁵S]-methionine and chased with excess unlabeled methionine for 0, 6, 12 and 24 hours, respectively. RB proteins were determined by immunoprecipitation.

The half-life of p94^{RB} protein in the transfected 5637 cells was determined to be 12 hours. In

contrast, the half-life of p110^{RB} protein was 4-6 hours. Therefore, p94^{RB} protein expressed in host tumor cells has a slower turnover, which is believed to contribute to its efficacy as a suppressor of both
5 RB+ and RB- tumor cell replication.

The comparative phosphorylation states of p110^{RB} and p94^{RB} in transiently transfected 5637 cells were determined by Western blot analysis: cell-lysates were made from WI-38, parental 5637 and pBA-f-RB33
10 (expressing p110^{RB}, Section 4.3.5) or pBA-s-RB34 (expressing p94^{RB}, Section 4.3.4) plasmid transfected 5637 cells approximately 24 hours after transfection. The basic protocol for Western blot analysis was described in Xu, H-J., et al., 1989, Oncogene, 4:807-
15 812. Each lane was loaded with 40μl of the lysate corresponding to 4 x 10⁵ cultured cells. Proteins were separated by 8% SDS-PAGE and electroblotted to a PVDF membrane. After blocking with 3% non-fat milk in TBST (10mM Tris-HCl, pH8.0, 150mM NaCl, 0.05% Tween 20),
20 the membrane was incubated with MAb-1 monoclonal anti-RB antibody at 0.1 μg/cm² overnight. The blot was then probed by the Enhanced Chemiluminescence (ECL) (Amersham Corporation, Arlington Heights, Illinois) immunodetection method. X-ray films were exposed for
25 2 seconds (Figure 10, lane 1) or 30 seconds (Figure 10, lanes 2-4).

Of particular interest was the fact that the RB-defective bladder carcinoma cell line, 5637, failed to phosphorylate the p94^{RB} protein as shown by Western
30 blot analysis (Figure 10, lane 4), although the p110^{RB} proteins expressed in transfected 5637 cells were fully phosphorylated (Figure 10, lane 3). Therefore, the presence of only unphosphorylated p94^{RB} proteins may also account for the failure of transfected 5637
35 tumor cells to enter S phase, and this in turn may cause cellular senescence and cell death.

4.6. Treatment of Human Bladder Cancers In Vivo.

The human bladder cancer represents an ideal model for practicing tumor suppressor gene therapy of solid tumors by infusing the p94^{RB} protein expression retroviral vectors into the bladder. The original experimental model of human bladder cancer was established by Dr. Peter A. Jones and his colleagues (Ahlering, T.E., et al., Cancer Res., 1987, 47:6660-6665). It has been shown that human bladder tumor cells of RT4 cell line established from a superficial papillary tumor (which usually does not metastasize) produced tumors only locally when injected by a 22-gauge catheter into the bladder of female nude mice. In contrast, the EJ bladder carcinoma cells which were originally isolated from a more aggressive human bladder cancer produced invasive tumors in the nude mouse bladders which metastasized to the lung spontaneously (Ahlering, T.E., et al., Cancer Res., 1987, 47:6660-6665). Therefore, this model can be used for treatment of experimental bladder cancer by in vivo gene transfer with retroviral vectors.

Tumor cells from RB minus human bladder carcinoma cell line, 5637 (ATCC HTB9) and RB⁺ human bladder carcinoma cell line, SCaBER (ATCC HTB3) are injected directly into the bladders of female athymic (nu/nu) nude mice (6 to 8 weeks of age) by a catheter as initially reported by Jones and his colleagues (Ahlering, T.E., et al., Cancer Res., 1987, 47:6660-6665).

Development and progression of the nude mouse bladder tumors are monitored using a fiber-optical system to which a TV monitor is attached. The experimental tumors are subsequently treated with retrovirus vectors expressing the p94^{RB}.

Supernatants with high virus titers are obtained from tissue culture media of selected PA317 clones

expressing high level of human p94^{RB} protein
(Section 4.3.7) and confirmed as free of replication-
competent virus prior to use. The retroviral vector
suspension at high titers ranging from 4×10^4 to
5 greater than 1×10^7 colony-forming unit (cfu)/ml, and
more preferably at a titer greater than 1×10^6 cfu/ml
is then infused directly into the mouse bladders via a
catheter to treat the tumors. The skilled artisan
will understand that such treatments can be repeated
10 as many times as necessary via a catheter inserted
into the bladder. The tumor regression following
transferring the p94^{RB} gene is monitored frequently via
the fiber-optic system mentioned above.

The same procedure as described above is used for
15 treating the human bladder cancer except that the
retroviral vector suspension is infused into a human
bladder bearing cancer.

20 4.7. In Vivo Studies Using an Orthotopic Lung Cancer Model

Human large cell lung carcinoma, NCI-H460 (ATCC
HTB177) cells which have normal p110^{RB} expression are
injected into the right mainstream bronchus of athymic
(nu/nu) nude mice (10^5 cells per mouse). Three days
25 later the mice are inoculated endobronchically with
supernatant from the p94^{RB}, or p110^{RB} retrovirus
producer cells daily for three consecutive days.
Tumor formation is suppressed in the group of mice
treated with the p94^{RB} retrovirus supernatant. In
30 contrast, in the other group, which is treated with
p110^{RB} retrovirus supernatant, the majority of mice
develop endobronchial tumors. This indicates that the
p94^{RB}-expressing retrovirus inhibits growth of RB+ non-
small cell lung carcinoma (NSCLC) cells, whereas the
35 p110^{RB}-expressing retrovirus does not.

4.8. Treatment of Human Non-Small Cell Lung Cancers In Vivo.

Non-small cell lung cancer patients having an endobronchial tumor accessible to a bronchoscope, and
5 also having a bronchial obstruction, are initially selected for p94^{RB} gene therapy. Treatment is administered by bronchoscopy under topical or general anesthesia. To begin the procedure, as much gross tumor as possible is resected endoscopically. A
10 transbronchial aspiration needle (21G) is passed through the biopsy channel of the bronchoscope.

The residual tumor site is injected with the appropriate retroviral vector supernatant (Section 4.3.7), adenovirus Ad-RB94 suspension
15 (Section 4.3.8) or p94^{RB}-expressing plasmid vector-liposome complexes (Section 4.3.4 and 4.3.6) at a volume of 5 ml to 10 ml. Protamine is added at a concentration of 5 µg/ml. The injections of therapeutic viral or plasmid supernatant comprising
20 one or more of the vectors are administered around and within the tumor or tumors and into the submucosa adjacent to the tumor. The injections are repeated daily for five consecutive days and monthly thereafter. The treatment may be continued as long as there is no
25 tumor progression. After one year the patients are evaluated to determine whether it is appropriate to continue therapy.

In addition, as a precaution the patients wear a surgical mask for 24 hours following injection of the
30 viral supernatant. All medical personnel wear masks routinely during bronchoscopy and injection of the viral supernatant. Anti-tussive is prescribed as necessary.

35 4.9 Treatment or Prevention of Human Lung Carcinomas With Liposome-Encapsulated Purified p94^{RB} Protein

In yet another alternative, target tumor or cancer cells are treated by introducing p94^{RB} protein into cells in need of such treatment by any known method. For example, liposomes are artificial
5 membrane vesicles that have been extensively studied for their usefulness as delivery vehicles of drugs, proteins and plasmid vectors both in vitro or in vivo (Mannino, R.J. et al., 1988, Biotechniques, 6:682-690). Proteins such as erythrocyte anion transporter
10 (Newton, A.C. and Huestis, W.H., Biochemistry, 1988, 27:4655-4659), superoxide dismutase and catalase (Tanswell, A.K. et al., 1990, Biochimica et Biophysica Acta, 1044:269-274), and UV-DNA repair enzyme (Ceccoll, J. et al. Journal of Investigative
15 Dermatology, 1989, 93:190-194) have been encapsulated at high efficiency with liposome vesicles and delivered into mammalian cells in vitro or in vivo.

Further, small-particle aerosols provide a method for the delivery of drugs for treatment of respiratory
20 diseases. For example, it has been reported that drugs can be administered in small-particle aerosols by using liposomes as a vehicle. Administered via aerosols, the drugs are deposited rather uniformly on the surface of the nasopharynx, the tracheobronchial
25 tree and in the pulmonary area (Knight, V. and Gilbert, B., 1988, European Journal of Clinical Microbiology and Infectious Diseases, 7:721-731).

To treat or prevent lung cancers, the therapeutic p94^{RB} protein is purified, for example, from
30 recombinant baculovirus AcMNPV-RB94 infected insect cells by immunoaffinity chromatography (Sections 4.1 and 4.2) or any other convenient source. The p94^{RB} protein is mixed with liposomes and incorporated into the liposome vesicles at high efficiency. The
35 encapsulated p94^{RB} is active. Since the aerosol delivery method is mild and well-tolerated by normal volunteers and patients, the p94^{RB}-containing liposomes

can be administered to treat patients suffering from lung cancers of any stage and/or to prevent lung cancers in high-risk population. The p94^{RB} protein-containing liposomes are administered by nasal
5 inhalation or by a endotracheal tube via small-particle aerosols at a dose sufficient to suppress abnormal cell proliferation. Aerosolization treatments are administered to a patient for 30 minutes, three times daily for two weeks, with
10 repetition as needed. The p94^{RB} protein is thereby delivered throughout the respiratory tract and the pulmonary area. The treatment may be continued as long as necessary. After one year the patient's overall condition will be evaluated to determine if
15 continued therapy is appropriate.

4.10 p94^{RB} Treatment is Non-Toxic to Normal Cells In Vitro

The retroviral vector, pLRB94RNL, expressing p94^{RB}
20 protein as described supra in section 4.3.7. was introduced into normal mouse fibroblast-derived retrovirus-packaging cell line, PA317 (ATCC CRL9078) by LIPOFECTIN reagent (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD). Single cell colonies were
25 isolated by selection in G418-containing medium and expanded into mass cultures. These clonal cells had been maintained over a one-year period of continuous culture, and stably expressed high levels of p94^{RB} protein as determined by immunocytochemical staining
30 (Figure 11) or by Western immunoblotting. These clones were indistinguishable from their parental normal mouse PA317 cells, or PA317 cells expressing human p110^{RB} protein in terms of morphology (Figure 11) and growth rate. The results indicate that p94^{RB}
35 protein expression was non-toxic to normal cells in vitro.

4.11 p94^{RB} Treatment is Non-Toxic to Normal Tissues In Vivo

The retroviral plasmid vector, pLRB94RNL, expressing p94^{RB} protein as described *supra* in section 4.3.7. was mixed with DMRIE/DOPE Liposomes (VICAL, Inc., San Diego, CA) and infused directly into the mouse urinary bladders via a catheter. Forty-eight hours after treatment, the mice were sacrificed and bladders excised. As demonstrated by immunohistochemical staining of the p94^{RB} protein in paraffin-embedded tissue sections from the mouse bladders (Figure 12), the liposome-encapsulated p94^{RB} expressing retroviral plasmid vectors penetrated the mucosa of mouse bladders and expressed p94^{RB} protein in the great majority of the transitional cells. The transitional epithelia expressing the p94^{RB} were histologically normal (Figure 12, panel D, arrows), and were indistinguishable from the mucosa in untreated mouse bladders or mouse bladders treated with liposomes only (Figure 12). The results from such animal experiments strongly suggest that the p94^{RB} treatment, unlike the conventional cytotoxic cancer therapy, is non-toxic to normal tissues *in vivo*.

25 5. Deposit of Microorganisms

The following were deposited on February 10, 1993 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852:

	<u>Escherichia coli</u> DH5α	<u>ATCC Designation</u>
30	DHC-S-RB42	69240
	DHB-S-RB34	69241

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such

modifications are intended to fall within the scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Xu, Hong-Ji
Hu, Shi-Xue
Benedict, William F.
- (ii) TITLE OF INVENTION: BROAD SPECTRUM TUMOR SUPPRESSOR GENES, GENE PRODUCTS, AND METHODS FOR TUMOR SUPPRESSION GENE THERAPY
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Pennie & Edmonds
 - (B) STREET: 1155 Avenue of the Americas
 - (C) CITY: New York
 - (D) STATE: New York
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 10036-2711
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: To be assigned
 - (B) FILING DATE: On even date herewith
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Poissant, Brian M
 - (B) REGISTRATION NUMBER: 28,462
 - (C) REFERENCE/DOCKET NUMBER: 7409-025-228
- (ix) TELECOMMUNICATION INFORMATION:
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 - (B) TELEFAX: (212) 869-9741/8864
 - (C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3232 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: not relevant
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 19..2469

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATCCCGACC TAGATGAG ATG TCG TTC ACT TTT ACT GAG CTA CAG AAA AAC
Met Ser Phe Thr Phe Thr Glu Leu Gln Lys Asn
1 5 10

-70-

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ACC AGT ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG TAT Thr Ser Thr Lys Val Asp Asn Ala Met Ser Arg Leu Leu Lys Lys Tyr 30 35 40	147
GAT GTA TTG TTT GCA CTC TTC AGC AAA TTG GAA AGG ACA TGT GAA CTT Asp Val Leu Phe Ala Leu Phe Ser Lys Leu Glu Arg Thr Cys Glu Leu 45 50 55	195
ATA TAT TTG ACA CAA CCC AGC AGT TCG ATA TCT ACT GAA ATA AAT TCT Ile Tyr Leu Thr Gln Pro Ser Ser Ser Ile Ser Thr Glu Ile Asn Ser 60 65 70 75	243
GCA TTG GTG CTA AAA GTT TCT TGG ATC ACA TTT TTA TTA GCT AAA GGG Ala Leu Val Leu Lys Val Ser Trp Ile Thr Phe Leu Leu Ala Lys Gly 80 85 90	291
GAA GTA TTA CAA ATG GAA GAT GAT CTG GTG ATT TCA TTT CAG TTA ATG Glu Val Leu Gln Met Glu Asp Asp Leu Val Ile Ser Phe Gln Leu Met 95 100 105	339
CTA TGT GTC CTT GAC TAT TTT ATT AAA CTC TCA CCT CCC ATG TTG CTC Leu Cys Val Leu Asp Tyr Phe Ile Lys Leu Ser Pro Pro Met Leu Leu 110 115 120	387
AAA GAA CCA TAT AAA ACA GCT GTT ATA CCC ATT AAT GGT TCA CCT CGA Lys Glu Pro Tyr Lys Thr Ala Val Ile Pro Ile Asn Gly Ser Pro Arg 125 130 135	435
ACA CCC AGG CGA GGT CAG AAC AGG AGT GCA CGG ATA GCA AAA CAA CTA Thr Pro Arg Arg Gly Gln Asn Arg Ser Ala Arg Ile Ala Lys Gln Leu 140 145 150 155	483
GAA AAT GAT ACA AGA ATT ATT GAA GTT CTC TGT AAA GAA CAT GAA TGT Glu Asn Asp Thr Arg Ile Ile Glu Val Leu Cys Lys Glu His Glu Cys 160 165 170	531
AAT ATA GAT GAG GTG AAA AAT GTT TAT TTC AAA AAT TTT ATA CCT TTT Asn Ile Asp Glu Val Lys Asn Val Tyr Phe Lys Asn Phe Ile Pro Phe 175 180 185	579
ATG AAT TCT CTT GGA CTT GTA ACA TCT AAT GGA CTT CCA GAG GTT GAA Met Asn Ser Leu Gly Leu Val Thr Ser Asn Gly Leu Pro Glu Val Glu 190 195 200	627
AAT CTT TCT AAA CGA TAC GAA GAA ATT TAT CTT AAA AAT AAA GAT CTA Asn Leu Ser Lys Arg Tyr Glu Glu Ile Tyr Leu Lys Asn Lys Asp Leu 205 210 215	675
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GAA GAG GTG AAT GTA ATT CCT CCA CAC ACT CCA GTT AGG ACT GTT ATG Glu Glu Val Asn Val Ile Pro Pro His Thr Pro Val Arg Thr Val Met 255 260 265	819
AAC ACT ATC CAA CAA TTA ATG ATG ATT TTA AAT TCA GCA AGT GAT CAA Asn Thr Ile Gln Gln Leu Met Met Ile Leu Asn Ser Ala Ser Asp Gln 270 275 280	867

CCT Pro	TCA Ser	GAA Glu	AAT Asn	CTG Leu	ATT Ile	TCC Ser	TAT Tyr	TTT Phe	AAC Asn	AAC Asn	TGC Cys	ACA Thr	GTG Val	AAT Asn	CCA Pro	915
285						290					295					
AAA Lys	GAA Glu	AGT Ser	ATA Ile	CTG Leu	AAA Lys	AGA Arg	GTG Val	AAG Lys	GAT Asp	ATA Ile	GGA Gly	TAC Tyr	ATC Ile	TTT Phe	AAA Lys	963
300					305					310					315	
GAG Glu	AAA Lys	TTT Phe	GCT Ala	AAA Lys	GCT Ala	GTG Val	GGA Gly	CAG Gln	GGT Gly	TGT Cys	GTC Val	GAA Glu	ATT Ile	GGA Gly	TCA Ser	1011
				320					325					330		
CAG Gln	CGA Arg	TAC Tyr	AAA Lys	CTT Leu	GGA Gly	GTT Val	CGC Arg	TTG Leu	TAT Tyr	TAC Tyr	CGA Arg	GTA Val	ATG Met	GAA Glu	TCC Ser	1059
			335					340					345			
ATG Met	CTT Leu	AAA Lys	TCA Ser	GAA Glu	GAA Glu	GAA Glu	CGA Arg	TTA Leu	TCC Ser	ATT Ile	CAA Gln	AAT Asn	TTT Phe	AGC Ser	AAA Lys	1107
		350					355					360				
CTT Leu	CTG Leu	AAT Asn	GAC Asp	AAC Asn	ATT Ile	TTT Phe	CAT His	ATG Met	TCT Ser	TTA Leu	TTG Leu	GCG Ala	TGC Cys	GCT Ala	CTT Leu	1155
		365				370					375					
GAG Glu	GTT Val	GTA Val	ATG Met	GCC Ala	ACA Thr	TAT Tyr	AGC Ser	AGA Arg	AGT Ser	ACA Thr	TCT Ser	CAG Gln	AAT Asn	CTT Leu	GAT Asp	1203
380					385					390					395	
TCT Ser	GGA Gly	ACA Thr	GAT Asp	TTG Leu	TCT Ser	TTC Phe	CCA Pro	TGG Trp	ATT Ile	CTG Leu	AAT Asn	GTG Val	CTT Leu	AAT Asn	TTA Leu	1251
				400					405					410		
AAA Lys	GCC Ala	TTT Phe	GAT Asp	TTT Phe	TAC Tyr	AAA Lys	GTG Val	ATC Ile	GAA Glu	AGT Ser	TTT Phe	ATC Ile	AAA Lys	GCA Ala	GAA Glu	1299
			415				420					425				
GGC Gly	AAC Asn	TTG Leu	ACA Thr	AGA Arg	GAA Glu	ATG Met	ATA Ile	AAA Lys	CAT His	TTA Leu	GAA Glu	CGA Arg	TGT Cys	GAA Glu	CAT His	1347
		430					435					440				
CGA Arg	ATC Ile	ATG Met	GAA Glu	TCC Ser	CTT Leu	GCA Ala	TGG Trp	CTC Leu	TCA Ser	GAT Asp	TCA Ser	CCT Pro	TTA Leu	TTT Phe	GAT Asp	1395
		445				450					455					
CTT Leu	ATT Ile	AAA Lys	CAA Gln	TCA Ser	AAG Lys	GAC Asp	CGA Arg	GAA Glu	GGA Gly	CCA Pro	ACT Thr	GAT Asp	CAC His	CTT Leu	GAA Glu	1443
460					465					470					475	
TCT Ser	GCT Ala	TGT Cys	CCT Pro	CTT Leu	AAT Asn	CTT Leu	CCT Pro	CTC Leu	CAG Gln	AAT Asn	AAT Asn	CAC His	ACT Thr	GCA Ala	GCA Ala	1491
				480					485					490		
GAT Asp	ATG Met	TAT Tyr	CTT Leu	TCT Ser	CCT Pro	GTA Val	AGA Arg	TCT Ser	CCA Pro	AAG Lys	AAA Lys	AAA Lys	GGT Gly	TCA Ser	ACT Thr	1539
			495					500					505			
ACG Thr	CGT Arg	GTA Val	AAT Asn	TCT Ser	ACT Thr	GCA Ala	AAT Asn	GCA Ala	GAG Glu	ACA Thr	CAA Gln	GCA Ala	ACC Thr	TCA Ser	GCC Ala	1587
		510					515					520				
TTC Phe	CAG Gln	ACC Thr	CAG Gln	AAG Lys	CCA Pro	TTG Leu	AAA Lys	TCT Ser	ACC Thr	TCT Ser	CTT Leu	TCA Ser	CTG Leu	TTT Phe	TAT Tyr	1635
	525					530					535					
AAA Lys	AAA Lys	GTG Val	TAT Tyr	CGG Arg	CTA Leu	GCC Ala	TAT Tyr	CTC Leu	CGG Arg	CTA Leu	AAT Asn	ACA Thr	CTT Leu	TGT Cys	GAA Glu	1683
540					545					550					555	

-72-

CGC CTT CTG TCT GAG CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC CTT	1731
Arg Leu Leu Ser Glu His Pro Glu Leu Glu His Ile Ile Trp Thr Leu	
560 565 570	
TTC CAG CAC ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG CAT	1779
Ph Gln His Thr Leu Gln Asn Glu Tyr Glu Leu Met Arg Asp Arg His	
575 580 585	
TTG GAC CAA ATT ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG AAG	1827
Leu Asp Gln Ile Met Met Cys Ser Met Tyr Gly Ile Cys Lys Val Lys	
590 595 600	
AAT ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC AAG GAT CTT	1875
Asn Ile Asp Leu Lys Phe Lys Ile Ile Val Thr Ala Tyr Lys Asp Leu	
605 610 615	
CCT CAT GCT GTT CAG GAG ACA TTC AAA CGT GTT TTG ATC AAA GAA GAG	1923
Pro His Ala Val Gln Glu Thr Phe Lys Arg Val Leu Ile Lys Glu Glu	
620 625 630 635	
GAG TAT GAT TCT ATT ATA GTA TTC TAT AAC TCG GTC TTC ATG CAG AGA	1971
Glu Tyr Asp Ser Ile Ile Val Phe Tyr Asn Ser Val Phe Met Gln Arg	
640 645 650	
CTG AAA ACA AAT ATT TTG CAG TAT GCT TCC ACC AGG CCC CCT ACC TTG	2019
Leu Lys Thr Asn Ile Leu Gln Tyr Ala Ser Thr Arg Pro Pro Thr Leu	
655 660 665	
TCA CCA ATA CCT CAC ATT CCT CGA AGC CCT TAC AAG TTT CCT AGT TCA	2067
Ser Pro Ile Pro His Ile Pro Arg Ser Pro Tyr Lys Phe Pro Ser Ser	
670 675 680	
CCC TTA CGG ATT CCT GGA GGG AAC ATC TAT ATT TCA CCC CTG AAG AGT	2115
Pro Leu Arg Ile Pro Gly Gly Asn Ile Tyr Ile Ser Pro Leu Lys Ser	
685 690 695	
CCA TAT AAA ATT TCA GAA GGT CTG CCA ACA CCA ACA AAA ATG ACT CCA	2163
Pro Tyr Lys Ile Ser Glu Gly Leu Pro Thr Pro Thr Lys Met Thr Pro	
700 705 710 715	
AGA TCA AGA ATC TTA GTA TCA ATT GGT GAA TCA TTC GGG ACT TCT GAG	2211
Arg Ser Arg Ile Leu Val Ser Ile Gly Glu Ser Phe Gly Thr Ser Glu	
720 725 730	
AAG TTC CAG AAA ATA AAT CAG ATG GTA TGT AAC AGC GAC CGT GTG CTC	2259
Lys Phe Gln Lys Ile Asn Gln Met Val Cys Asn Ser Asp Arg Val Leu	
735 740 745	
AAA AGA AGT GCT GAA GGA AGC AAC CCT CCT AAA CCA CTG AAA AAA CTA	2307
Lys Arg Ser Ala Glu Gly Ser Asn Pro Pro Lys Pro Leu Lys Lys Leu	
750 755 760	
CGC TTT GAT ATT GAA GGA TCA GAT GAA GCA GAT GGA AGT AAA CAT CTC	2355
Arg Phe Asp Ile Glu Gly Ser Asp Glu Ala Asp Gly Ser Lys His Leu	
765 770 775	
CCA GGA GAG TCC AAA TTT CAG CAG AAA CTG GCA GAA ATG ACT TCT ACT	2403
Pro Gly Glu Ser Lys Phe Gln Gln Lys Leu Ala Glu Met Thr Ser Thr	
780 785 790 795	
CGA ACA CGA ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC TCA	2451
Arg Thr Arg Met Gln Lys Gln Lys Met Asn Asp Ser Met Asp Thr Ser	
800 805 810	
AAC AAG GAA GAG AAA TGAGGATCTC AGGACCTTGG TGGACACTGT GTACACCTCT	2506
Asn Lys Glu Glu Lys	
815	

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GGATTCATTG TCTCTCACAG ATGTGACTGT ATAACCTTCC CAGGTTCTGT TTATGGCCAC	2566
ATTTAATATC TTCAGCTCTT TTTGTGGATA TAAAATGTGC AGATGCAATT GTTTGGGTGA	2626
TTCCTAAGCC ACTTGAAATG TTAGTCATTG TTATTTATAC AAGATTGAAA ATCTTGTGTA	2686
AATCCTGCCA TTTAAAAAGT TGTAGCAGAT TGTTTCCTCT TCCAAAGTAA AATTGCTGTG	2746
CTTTATGGAT AGTAAGAATG GCCCTAGAGT GGGAGTCCTG ATAACCCAGG CCTGTCTGAC	2806
TACTTTGCCT TCTTTTGTAG CATATAGGTG ATGTTTGCTC TTGTTTTTAT TAATTTATAT	2866
GTATATTTTT TTAATTTAAC ATGAACACCC TTAGAAAATG TGTCCATCT ATCATCCAAA	2926
TGCAATTTGA TTGACTGCCC ATTCACCAAA ATTATCCTGA ACTCTTCTGC AAAAATGGAT	2986
ATTATTAGAA ATTAGAAAAA AATTACTAAT TTTACACATT AGATTTTATT TTACTATTGG	3046
AATCTGATAT ACTGTGTGCT TGTTTTATAA AATTTTGCTT TTAATTAAAT AAAAGCTGGA	3106
AGCAAAGTAT AACCATATGA TACTATCATA CTACTGAAAC AGATTTTCATA CCTCAGAATG	3166
TAAAAGAACT TACTGATTAT TTTCTTCATC CAACTTATGT TTTTAAATGA GGATTATTGA	3226
TAGTGG	3232

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 3232 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCCCACTA TCAATAATCC TCATTTAAAA ACATAAGTTG GATGAAGAAA ATAATCAGTA	60
AGTTCTTTTA CATTCTGAGG TATGAAATCT GTTTCAGTAG TATGATAGTA TCATATGGTT	120
ATACTTTGCT TCCAGCTTTT ATTTAATTAA AAGCAAATT TTATAAAACA AGCACACAGT	180
ATATCAGATT CCAATAGTAA AATAAAATCT AATGTGTAAT ATTAGTAATT TTTTCTAAT	240
TTCTAATAAT ATCCATTTTT GCAGAAGAGT TCAGGATAAT TTTGGTGAAT GGGCAGTCAA	300
TCAAATTGCA TTTGGATGAT AGATAGGACA CATTTTCTAA GGGTGTTTAT GTTAAATTAA	360
AAAAATATAC ATATAAATTA ATAAAAACAA GAGCAAACAT CACCTATATG CTACAAAAGA	420
AGGCAAAGTA GTCAGACAGG CCTGGGTTAT CAGGACTCCC ACTCTAGGGC CATTCTTACT	480
ATCCATAAAG CACAGCAATT TTACTTTGGA AGAGGAAACA ATCTGCTACA ACTTTTTAAA	540
TGGCAGGATT TACACAAGAT TTTCAATCTT GTATAAATAA CAATGACTAA CATTTCAAGT	600
GGCTTAGGAA TCACCCAAAC AATTGCATCT GCACATTTTA TATCCACAAA AAGAGCTGAA	660
GATATTAAAT GTGGCCATAA ACAGAACCTG GGAAAGTTAT ACAGTCACAT CTGTGAGAGA	720
CAATGAATCC AGAGGTGTAC ACAGTGTCCA CCAAGGTCCT GAGATCCTCA TTTCTCTTCC	780

TTGTTTGAGG TATCCATGCT ATCATTCAAT TTCTGCTTTT GCATTTCGTGT TCGAGTAGAA	840
GTCATTTCTG CCAGTTTCTG CTGAAATTTG GACTCTCCTG GGAGATGTTT ACTTCCATCT	900
GCTTCATCTG ATCCTTCAAT ATCAAAGCGT AGTTTTTTCA GTGGTTTAGG AGGGTTGCTT	960
CCTTCAGCAC TTCTTTTGAG CACACGGTCG CTGTTACATA CCATCTGATT TATTTTCTGG	1020
AACTTCTCAG AAGTCCCGAA TGATTCACCA ATTGATACTA AGATTCTTGA TCTTGGAGTC	1080
ATTTTTGTTG GTGTTGGCAG ACCTTCTGAA ATTTTATATG GACTCTTCAG GGGTGAAATA	1140
TAGATGTTCC CTCCAGGAAT CCGTAAGGGT GAACTAGGAA ACTTGTAAGG GCTTCGAGGA	1200
ATGTGAGGTA TTGGTGACAA GGTAGGGGGC CTGGTGGAAG CATACTGCAA AATATTTGTT	1260
TTCAGTCTCT GCATGAAGAC CGAGTTATAG AATACTATAA TAGAATCATA CTCCTCTTCT	1320
TTGATCAAAA CACGTTTGAA TGTCTCCTGA ACAGCATGAG GAAGATCCTT GTATGCTGTT	1380
ACAATGATTT TGAATTTAAG GTCTATATTC TTCACTTTGC ATATGCCATA CATGGAACAC	1440
ATCATAATTT GGTCCAAATG CCTGTCTCTC ATGAGTTCAT ACTCATTCTG CAGGGTGTGC	1500
TGGAAAAGGG TCCAGATGAT ATGTTCTAAT TCTGGGTGCT CAGACAGAAG GCGTTCACAA	1560
AGTGTATTTA GCCGGAGATA GGCTAGCCGA TACACTTTTT TATAAACAG TGAAAGAGAG	1620
GTAGATTTCA ATGGCTTCTG GGTCTGGAAG GCTGAGGTTG CTTGTGTCTC TGCATTTGCA	1680
GTAGAATTTA CACGCGTAGT TGAACCTTTT TTCTTTGGAG ATCTTACAGG AGAAAGATAC	1740
ATATCTGCTG CAGTGTGATT ATTCTGGAGA GGAAGATTAA GAGGACAAGC AGATTCAAGG	1800
TGATCAGTTG GTCCTTCTCG GTCCTTTGAT TGTTTAATAA GATCAAATAA AGGTGAATCT	1860
GAGAGCCATG CAAGGGATTG CATGATTGCA TGTTACATC GTTCTAAATG TTTTATCATT	1920
TCTCTTGTC AATTGTCCTT TGCTTTGATA AAACCTTCGA TCACTTTGTA AAAATCAAAG	1980
GCTTTTAAAT TAAGCACATT CAGAATCCAT GGGAAAGACA AATCTGTTCC AGAATCAAGA	2040
TTCTGAGATG TACTTCTGCT ATATGTGGCC ATTACAACCT CAAGAGCGCA CGCCAATAAA	2100
GACATATGAA AAATGTTGTC ATTCAGAAGT TTGCTAAAAT TTTGAATGGA TAATCGTTCT	2160
TCTTCTGATT TAAGCATGGA TTCCATTACT CGGTAATACA AGCGAACTCC AAGTTTGTAT	2220
CGCTGTGATC CAATTTGAC ACAACCCTGT CCCACAGCTT TAGCAAATTT CTCTTTAAAG	2280
ATGTATCCTA TATCCTTCAC TCTTTTCAGT ATACTTTCTT TTGGATTAC TGTGCAGTTG	2340
TTAAATAGG AAATCAGATT TTCTGAAGGT TGATCACTTG CTGAATTTAA AATCATCATT	2400
AATTGTTGGA TAGTGTTTAT AACAGTCCTA ACTGGAGTGT GTGGAGGAAT TACATTCACC	2460
TCTTCATCAA GGTTACTTTT TCGTGGTGTT CTCTGTGTTT CAAACTGTC TATAGAATCA	2520
GTCTGAAGAG TTTTATCATG ATCCAAAAT AATCTGTCAT CTAGATCTTT ATTTTAAAGA	2580
TAAATTTCTT CGTATCGTTT AGAAAGATTT TCAACCTCTG GAAGTCCATT AGATGTTACA	2640
AGTCCAAGAG AATTCATAAA AGGTATAAAA TTTTGAAT AAACATTTTT CACCTCATCT	2700
ATATTACATT CATGTTCTTT ACAGAGAACT TCAATAATTC TTGTATCATT TTCTAGTTGT	2760
TTTGCTATCC GTGCACTCCT GTTCTGACCT CGCCTGGGTG TTCGAGGTGA ACCATTAATG	2820

GGTATAACAG CTGTTTTATA TGGTTCTTTG AGCAACATGG GAGGTGAGAG TTTAATAAAA 2880
 TAGTCAAGGA CACATAGCAT TAACTGAAAT GAAATCACCA GATCATCTTC CATTTGTAAT 2940
 ACTTCCCCTT TAGCTAATAA AAATGTGATC CAAGAAACTT TTAGCACCAA TGCAGAATTT 3000
 ATTTCAAGTAG ATATCGAACT GCTGGGTTGT GTCAAATATA TAAGTTCACA TGTCTTTTCC 3060
 AATTTGCTGA AGAGTGCAA CAATACATCA TACTTCTTCA ACAGTCTTGA CATAGCATT 3120
 TCAACTTTGG TACTGGTATC AATTTCTTTT AGTAAGTTAA AGAATTTATG GACACTGATT 3180
 TCTATGTTTT TCTGTAGCTC AGTAAAAGTG AACGACATCT CATCTAGGTC GG 3232

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 816 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Ser	Phe	Thr	Phe	Thr	Glu	Leu	Gln	Lys	Asn	Ile	Glu	Ile	Ser	Val	1	5	10	15
His	Lys	Phe	Phe	Asn	Leu	Leu	Lys	Glu	Ile	Asp	Thr	Ser	Thr	Lys	Val	20	25	30	
Asp	Asn	Ala	Met	Ser	Arg	Leu	Leu	Lys	Lys	Tyr	Asp	Val	Leu	Phe	Ala	35	40	45	
Leu	Phe	Ser	Lys	Leu	Glu	Arg	Thr	Cys	Glu	Leu	Ile	Tyr	Leu	Thr	Gln	50	55	60	
Pro	Ser	Ser	Ser	Ile	Ser	Thr	Glu	Ile	Asn	Ser	Ala	Leu	Val	Leu	Lys	65	70	75	80
Val	Ser	Trp	Ile	Thr	Phe	Leu	Leu	Ala	Lys	Gly	Glu	Val	Leu	Gln	Met	85	90	95	
Glu	Asp	Asp	Leu	Val	Ile	Ser	Phe	Gln	Leu	Met	Leu	Cys	Val	Leu	Asp	100	105	110	
Tyr	Phe	Ile	Lys	Leu	Ser	Pro	Pro	Met	Leu	Leu	Lys	Glu	Pro	Tyr	Lys	115	120	125	
Thr	Ala	Val	Ile	Pro	Ile	Asn	Gly	Ser	Pro	Arg	Thr	Pro	Arg	Arg	Gly	130	135	140	
Gln	Asn	Arg	Ser	Ala	Arg	Ile	Ala	Lys	Gln	Leu	Glu	Asn	Asp	Thr	Arg	145	150	155	160
Ile	Ile	Glu	Val	Leu	Cys	Lys	Glu	His	Glu	Cys	Asn	Ile	Asp	Glu	Val	165	170	175	
Lys	Asn	Val	Tyr	Phe	Lys	Asn	Phe	Ile	Pro	Phe	Met	Asn	Ser	Leu	Gly	180	185	190	
Leu	Val	Thr	Ser	Asn	Gly	Leu	Pro	Glu	Val	Glu	Asn	Leu	Ser	Lys	Arg	195	200	205	
Tyr	Glu	Glu	Ile	Tyr	Leu	Lys	Asn	Lys	Asp	Leu	Asp	Ala	Arg	Leu	Phe	210	215	220	

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Leu Asp His Asp Lys Thr Leu Gln Thr Asp Ser Ile Asp Ser Phe Glu
 225 230 235 240
 Thr Gln Arg Thr Pro Arg Lys Ser Asn Leu Asp Glu Glu Val Asn Val
 245 250 255
 Ile Pro Pro His Thr Pro Val Arg Thr Val Met Asn Thr Ile Gln Gln
 260 265 270
 Leu Met Met Ile Leu Asn Ser Ala Ser Asp Gln Pro Ser Glu Asn Leu
 275 280 285
 Ile Ser Tyr Phe Asn Asn Cys Thr Val Asn Pro Lys Glu Ser Ile Leu
 290 295 300
 Lys Arg Val Lys Asp Ile Gly Tyr Ile Phe Lys Glu Lys Phe Ala Lys
 305 310 315 320
 Ala Val Gly Gln Gly Cys Val Glu Ile Gly Ser Gln Arg Tyr Lys Leu
 325 330 335
 Gly Val Arg Leu Tyr Tyr Arg Val Met Glu Ser Met Leu Lys Ser Glu
 340 345 350
 Glu Glu Arg Leu Ser Ile Gln Asn Phe Ser Lys Leu Leu Asn Asp Asn
 355 360 365
 Ile Phe His Met Ser Leu Leu Ala Cys Ala Leu Glu Val Val Met Ala
 370 375 380
 Thr Tyr Ser Arg Ser Thr Ser Gln Asn Leu Asp Ser Gly Thr Asp Leu
 385 390 395 400
 Ser Phe Pro Trp Ile Leu Asn Val Leu Asn Leu Lys Ala Phe Asp Phe
 405 410 415
 Tyr Lys Val Ile Glu Ser Phe Ile Lys Ala Glu Gly Asn Leu Thr Arg
 420 425 430
 Glu Met Ile Lys His Leu Glu Arg Cys Glu His Arg Ile Met Glu Ser
 435 440 445
 Leu Ala Trp Leu Ser Asp Ser Pro Leu Phe Asp Leu Ile Lys Gln Ser
 450 455 460
 Lys Asp Arg Glu Gly Pro Thr Asp His Leu Glu Ser Ala Cys Pro Leu
 465 470 475 480
 Asn Leu Pro Leu Gln Asn Asn His Thr Ala Ala Asp Met Tyr Leu Ser
 485 490 495
 Pro Val Arg Ser Pro Lys Lys Lys Gly Ser Thr Thr Arg Val Asn Ser
 500 505 510
 Thr Ala Asn Ala Glu Thr Gln Ala Thr Ser Ala Phe Gln Thr Gln Lys
 515 520 525
 Pro Leu Lys Ser Thr Ser Leu Ser Leu Phe Tyr Lys Lys Val Tyr Arg
 530 535 540
 Leu Ala Tyr Leu Arg Leu Asn Thr Leu Cys Glu Arg Leu Leu Ser Glu
 545 550 555 560
 His Pro Glu Leu Glu His Ile Ile Trp Thr Leu Phe Gln His Thr Leu
 565 570 575
 Gln Asn Glu Tyr Glu Leu Met Arg Asp Arg His Leu Asp Gln Ile Met

580							585							590						
Met	Cys	Ser	Met	Tyr	Gly	Ile	Cys	Lys	Val	Lys	Asn	Ile	Asp	Leu	Lys					
		595					600					605								
Phe	Lys	Ile	Ile	Val	Thr	Ala	Tyr	Lys	Asp	Leu	Pro	His	Ala	Val	Gln					
	610					615					620									
Glu	Thr	Phe	Lys	Arg	Val	Leu	Ile	Lys	Glu	Glu	Glu	Tyr	Asp	Ser	Ile					
	625				630					635					640					
Ile	Val	Phe	Tyr	Asn	Ser	Val	Phe	Met	Gln	Arg	Leu	Lys	Thr	Asn	Ile					
				645					650					655						
Leu	Gln	Tyr	Ala	Ser	Thr	Arg	Pro	Pro	Thr	Leu	Ser	Pro	Ile	Pro	His					
			660				665						670							
Ile	Pro	Arg	Ser	Pro	Tyr	Lys	Phe	Pro	Ser	Ser	Pro	Leu	Arg	Ile	Pro					
		675					680					685								
Gly	Gly	Asn	Ile	Tyr	Ile	Ser	Pro	Leu	Lys	Ser	Pro	Tyr	Lys	Ile	Ser					
	690					695					700									
Glu	Gly	Leu	Pro	Thr	Pro	Thr	Lys	Met	Thr	Pro	Arg	Ser	Arg	Ile	Leu					
	705				710					715					720					
Val	Ser	Ile	Gly	Glu	Ser	Phe	Gly	Thr	Ser	Glu	Lys	Phe	Gln	Lys	Ile					
				725					730					735						
Asn	Gln	Met	Val	Cys	Asn	Ser	Asp	Arg	Val	Leu	Lys	Arg	Ser	Ala	Glu					
			740				745						750							
Gly	Ser	Asn	Pro	Pro	Lys	Pro	Leu	Lys	Lys	Leu	Arg	Phe	Asp	Ile	Glu					
		755					760					765								
Gly	Ser	Asp	Glu	Ala	Asp	Gly	Ser	Lys	His	Leu	Pro	Gly	Glu	Ser	Lys					
	770					775					780									
Phe	Gln	Gln	Lys	Leu	Ala	Glu	Met	Thr	Ser	Thr	Arg	Thr	Arg	Met	Gln					
	785				790					795					800					
Lys	Gln	Lys	Met	Asn	Asp	Ser	Met	Asp	Thr	Ser	Asn	Lys	Glu	Glu	Lys					
				805					810					815						

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International Application No: PCT/

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 67, lines 25-37 of the description *

A. IDENTIFICATION OF DEPOSIT *

Further deposits are identified on an additional sheet *

Name of depositary institution *

American Type Culture Collection

Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit * February 10, 1993 Accession Number * 69240

B. ADDITIONAL INDICATIONS * (leave blank if not applicable) This information is continued on a separate attached sheet**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *** (if the indications are not all designated States)**D. SEPARATE FURNISHING OF INDICATIONS *** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the International application when filed (to be checked by the receiving Office)
(Authorized Officer)☐ The date of receipt (from the applicant) by the International Bureau *

was

(Authorized Officer)

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

American Type Culture Collection

**12301 Parklawn Drive
Rockville, MD 20852
US**

Accession No.

69241

Date of Deposit

February 10, 1993

We claim:

1. A method of treating a disease characterized by abnormal cellular proliferation in a mammal by a process comprising the steps of:
 - 5 a. administering an effective dose of a p94^{RB} encoding expression vector to a mammal having a disease characterized by abnormally proliferating cells, such that said expression vector is inserted into said abnormally proliferating cells, said
10 expression vector comprising a gene encoding p94^{RB}; and
 - b. expressing p94^{RB} in said abnormally proliferating cells in an amount effective to suppress proliferation of said abnormally proliferating cells; and wherein said p94^{RB} encoding expression vector
15 comprises a p94^{RB} encoding gene, said gene encoding a protein having an amino acid sequence substantially according to SEQ ID NO:3, provided that said protein is not p110^{RB}.
- 20 2. The method according to claim 1 wherein said p94^{RB} encoding gene encodes a protein having an amino acid sequence according to SEQ ID NO:3.
3. The method according to claim 1 wherein said
25 p94^{RB} encoding gene has a DNA sequence substantially according to SEQ ID NO:1.
4. The method according to claim 3 wherein said p94^{RB} encoding gene has a DNA sequence according to SEQ
30 ID NO:1.
5. The method according to claim 2 wherein said expression vector is selected from the group consisting of a plasmid and a viral vector.

6. The method according to claim 4 wherein said expression vector is selected from the group consisting of a plasmid and a viral vector.

5 7. The method according to claim 5 wherein said viral vector is selected from a group consisting of a retroviral vector, an adenoviral vector and a herpesviral vector, wherein said p94^{RB} encoding gene is under the control of a promoter selected from the
10 group consisting of a retroviral promoter, an adenoviral promoter, a CMV promoter and a β -actin promoter.

8. The method according to claim 6 wherein said
15 viral vector is selected from a group consisting of a retroviral vector, an adenoviral vector and a herpesviral vector wherein said p94^{RB} encoding gene is under the control of a promoter selected from the group consisting of a retroviral promoter, an
20 adenoviral promoter, a CMV promoter and a β -actin promoter.

9. The method according to claim 8 wherein said expression vector is selected from the group
25 consisting of plasmid pCMV-s-RB42 and plasmid p β A-s-RB34.

10. The method according to claims 7, 8 or 9 wherein said expression vector is inserted into said
30 abnormally proliferating cells by a method selected from the group consisting of viral infection or transduction, liposome-mediated transfection, polybrene-mediated transfection and CaPO₄ mediated transfection.

11. The method according to claim 1 wherein said abnormally proliferating cells are tumor or cancer cells and said mammal is a human.

5 12. The method according to claim 11 wherein said tumor or cancer cells are selected from the group consisting of carcinoma and sarcoma cells.

10 13. The method according to claim 11 wherein tumor or cancer cells are selected from the group consisting of a bladder carcinoma, a lung carcinoma, a breast carcinoma, a prostate carcinoma, a fibrosarcoma, an osteosarcoma, and a cervical carcinoma.

15 14. The method according to claim 11 wherein said tumor or cancer cells are cells having at least one genetically defective tumor suppressor gene or oncogene selected from the group consisting of an RB,
20 a p53, a c-myc an N-ras and a c-yes-1 gene.

15 15. The method according to claim 11 wherein said tumor or cancer cells have no detectable genetic defect of a tumor suppressor gene, and the tumor
25 suppressor gene is selected from the group consisting of an RB gene and a p53 gene.

30 16. The method according to claim 12 wherein said carcinoma cells are bladder carcinoma cells and said step of administering said expression vector to treat said bladder carcinoma cells is by means of an infusion of said expression vector into a bladder in need of such treatment.

35 17. A DNA molecule encoding p94^{RB} having an amino acid sequence substantially according to SEQ ID NO:3,

provided that said DNA molecule does not also code for p110^{RB}.

18. The DNA molecule according to claim 17, said
5 DNA molecule coding for a protein having an amino acid
sequence according to SEQ ID NO:3.

19. The DNA molecule according to claim 17, said
DNA molecule having a DNA sequence substantially
10 according to SEQ ID NO:1, provided that said DNA
molecule does not also code for p110^{RB}.

20. The DNA molecule according to claim 17, said
DNA molecule having a DNA sequence according to SEQ ID
15 NO:1.

21. An expression vector comprising said DNA
molecule according to claim 18, capable of inserting
said p94^{RB} encoding DNA molecule into a mammalian host
20 cell and of expressing p94^{RB} therein.

22. An expression vector comprising said DNA
molecule according to claim 20, capable of inserting
said p94^{RB} encoding DNA molecule into a mammalian host
25 cell and of expressing p94^{RB} therein.

23. The expression vector according to claim 21,
wherein said expression vector is selected from the
group consisting of a plasmid and a viral vector.
30

24. The expression vector according to claim 22,
wherein said expression vector is selected from the
group consisting of a plasmid and a viral vector.

35 25. The expression vector according to claim 23
wherein said viral vector is selected from a group
consisting of a retroviral vector, an adenoviral

vector and a herpesviral vector, and wherein said p94^{RB} encoding gene is under the control of a promoter selected from the group consisting of a retroviral promoter, an adenoviral promoter, a CMV promoter and a
5 β-actin promoter.

26. The expression vector according to claim 24 wherein said viral vector is selected from a group consisting of a retroviral vector, an adenoviral
10 vector and a herpesviral vector and wherein said p94^{RB} encoding gene is under the control of a promoter selected from the group consisting of a retroviral promoter, an adenoviral promoter, a CMV promoter and a β-actin promoter.

15

27. The expression vector according to claim 24 wherein said expression vector is plasmid pCMV-s-RB42 and plasmid pBA-s-RB34.

20 28. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of the expression vector according to claim 21, together with a suitable carrier or vehicle.

25 29. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of the expression vector according to claim 22, together with a suitable carrier or vehicle.

30 30. A composition according to claims 28 or 29 wherein said carrier or vehicle comprises an encapsulating liposome.

31. A p94^{RB} protein comprising a polypeptide
35 having an amino acid sequence substantially according to SEQ ID NO:3; provided that said protein is not p110^{RB}.

32. A p94^{RB} protein according to claim 31 comprising a polypeptide having an amino acid sequence according to SEQ ID NO:3.

5 33. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of p94^{RB} according to claim 31, together with a suitable carrier or vehicle.

10 34. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of p94^{RB} according to claim 32, together with a suitable carrier or vehicle.

15 35. A composition according to claims 33 or 34 wherein said carrier or vehicle comprises an encapsulating liposome.

20 36. A method of producing a p94^{RB} protein comprising the steps of:

- a. inserting a compatible expression vector comprising a p94^{RB} encoding gene into a host cell; and
 - b. causing said host cell to express p94^{RB}
- 25 protein.

30 37. The method according to claim 36 wherein said host cell is selected from the group consisting of a prokaryotic host cell and a eukaryotic host cell.

35 38. The method according to claim 32 wherein said eukaryotic host cell is a mammalian host cell and said expression vector is compatible with said mammalian host cell.

39. The method according to claim 38 wherein said expression vector is selected from the group

consisting of plasmid pCMV-s-RB42 and plasmid pBA-s-RB34.

40. The method according to claim 37 wherein
5 said host cell is an insect host cell and said
expression vector is a plasmid or a viral vector
compatible with said insect host cell.

41. The method according to claim 40 wherein
10 said baculovirus vector is AcMNPV-RB94.

42. A method of treating abnormally
proliferating cells of a mammal ex vivo by a process
comprising the steps of:

15 a. removing a tissue sample in need of
treatment from a mammal, said tissue sample comprising
abnormally proliferating cells;

b. contacting said tissue sample in need
of treatment with an effective dose of a p94^{RB} encoding
20 expression vector;

c. expressing said p94^{RB} in said abnormally
proliferating cells in amounts effective to suppress
proliferation of said abnormally proliferating cells;
and

25 d. returning said treated tissue sample to
said mammal or placing said tissue sample into another
mammal.

43. A method of treating a disease characterized
30 by abnormal cellular proliferation in a mammal by
administering p94^{RB} protein to a mammal having a
disease characterized by abnormally proliferating
cells, such that said p94^{RB} protein is inserted into
said abnormally proliferating cells in amounts
35 effective to suppress abnormal proliferation of said
cells.

44. The method according to claim 43 wherein said p94^{RB} protein has an amino acid sequence substantially according to SEQ ID NO:3, provided that said protein is not p110^{RB}.

5

45. The method according to claim 43 wherein said p94^{RB} protein has an amino acid sequence according to SEQ ID NO:3.

10 46. The method according to claim 43 wherein said abnormally proliferating cells are tumor or cancer cells, and said mammal is a human patient.

15 47. The method according to claim 46 wherein said p94^{RB} protein is encapsulated in a liposome carrier and said p94^{RB} protein is inserted into said abnormally proliferating cells by fusion of said liposome encapsulated p94^{RB} protein with said abnormally proliferating cells.

20

48. The method according to claim 46 wherein said tumor or cancer cells are selected from the group consisting of a bladder carcinoma, a lung carcinoma, a breast carcinoma, a prostate carcinoma, a
25 fibrosarcoma, an osteosarcoma, and a cervix carcinoma.

49. The method according to claim 46 wherein said tumor or cancer cells are cells having one or more genetically defective tumor suppressor genes and
30 oncogenes selected from the group consisting of an RB, a p53, a c-myc, an N-ras and a c-yes-1 gene.

50. The method according to claim 46 wherein said tumor or cancer cells are cells having no
35 detectable genetic defect of a tumor suppressor gene selected from the group consisting of an RB gene and a p53 gene.

51. The method according to claim 48 wherein said tumor or cancer cells are lung carcinoma cells and said step of administering said p94^{RB} protein to treat said lung carcinoma cells is by means of an
5 infusion of said liposome-encapsulated p94^{RB} protein into the respiratory tract and the pulmonary area in need of such treatment.

52. A method of treating abnormally
10 proliferating cells of a mammal ex vivo by a process comprising the steps of:

- a. removing a tissue sample in need of treatment from a mammal, said tissue sample comprising abnormally proliferating cells;
- 15 b. contacting said tissue sample in need of treatment with an effective dose of a p94^{RB} protein; and
- c. returning said treated tissue sample to said mammal or placing said tissue sample into another
20 mammal; and said p94^{RB} protein has the amino acid sequence substantially according to SEQ ID NO:3.

53. A method of treating abnormally
proliferating cells by a process comprising the steps
25 of:

- a. inserting a p94^{RB} encoding expression vector into abnormally proliferating cells of a mammal; and
- b. expressing said p94^{RB} therein in amounts
30 effective to suppress proliferation of said abnormally proliferating cells and said p94^{RB} encoding expression vector comprises a p94^{RB} encoding gene, said gene encoding a protein having an amino acid sequence substantially according to SEQ ID NO:3.

35

AMENDED CLAIMS

[received by the International Bureau on 11 August 1994 (11.08.94);
original claim 38 amended; remaining claims unchanged (1 page)]

32. A p94^{RB} protein according to claim 31
comprising a polypeptide having an amino acid sequence
according to SEQ ID NO:3.

5

33. A composition suitable for treating a tumor
or cancer in a mammal comprising an effective amount
of p94^{RB} according to claim 31, together with a
suitable carrier or vehicle.

10

34. A composition suitable for treating a tumor
or cancer in a mammal comprising an effective amount
of p94^{RB} according to claim 32, together with a
suitable carrier or vehicle.

15

35. A composition according to claims 33 or 34
wherein said carrier or vehicle comprises an
encapsulating liposome.

20

36. A method of producing a p94^{RB} protein
comprising the steps of:

a. inserting a compatible expression
vector comprising a p94^{RB} encoding gene into a host
cell; and

25

b. causing said host cell to express p94^{RB}
protein.

37. The method according to claim 36 wherein
said host cell is selected from the group consisting
30 of a prokaryotic host cell and a eukaryotic host cell.

38. The method according to claim 37 wherein
said eukaryotic host cell is a mammalian host cell and
said expression vector is compatible with said
35 mammalian host cell.

39. The method according to claim 38 wherein
said expression vector is selected from the group

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5' GATCCCGACC 10 TAGATGAGAT 20 GTCGTTCACT 30 TTTACTGAGC 40 TACAGAAAAA 50 CATAGAAATC 60
3' GGCTGG 70 ATCTACTCTA 80 CAGCAAGTGA 90 AAATGACTCG 100 GAAATGATA 110 CCAGTACCAA 120 AGTTGATAAT
130 GCTATGTCAA 140 GACTGTTGAA 150 GAATGATTTT 160 CTTTAACTAT 170 GGTCATGGTT 180 TCAACTATTA
190 CGATACAGTT 200 CTGACAACTT 210 CTTCACTACTA 220 CATAACAAAC 230 CACTCTTCAG 240 CAAATTGGAA
250 AGGACATGTG 260 AACTTATATA 270 TTTGACACAA 280 CCCAGCAGTT 290 CGATATCTAC 300 TGAAATAAAT
310 TCCTGTACAC 320 TTGAATATAT 330 AACTGTGTT 340 GGGTCGTCAA 350 GCTATAGATG 360 ACTTTATTTA
370 TCTGCATTGG 380 TGCTAAAAGT 390 TTCTTGGATC 400 ACATTTTAT 410 TAAGCTAAAGG 420 GGAAAGTATTA
430 AGACGTAACC 440 ACGATTTTCA 450 AAGAACCTAG 460 TGTAATAATA 470 ATCGATTTC 480 CCTTCATAAT
490 CAAATGGAAG 500 ATGATCTGGT 510 GATTTCATTT 520 CAGTTAATGC 530 TAAGTGTCCT 540 TGACTATTTT
550 GTTTACCTTC 560 TACTAGACCA 570 CTAAGTAAA 580 GTCAATTACG 590 ATACACAGGA 600 ACTGATAAAA
610 ATTAACCTCT 620 CACCTCCCAT 630 GTTGCTCAA 640 GAACCATATA 650 AAACAGCTGT 660 TATACCCATT
670 TAATTTGAGA 680 GTGGAGGTA 690 CAACGAGTTT 700 CTTGGTATAT 710 TTTGTCGACA 720 ATATGGGTAA
730 AATGGTTCAC 740 CTCGAACACC 750 CAGGCGAGGT 760 CAGAACAGGA 770 GTGCACGGAT 780 AGCAAAACAA
790 TTACCAAGTG 800 GAGCTTGTGG 810 GTCCGCTCCA 820 GTCTTGTCCT 830 CAGGTGCCTA 840 TCGTTTGT
850 CTAGAAAATG 860 ATACAAGAAT 870 TATTGAAGTT 880 CTCTGTAAG 890 AACATGAATG 900 TAATATAGAT
910 GATCTTTTAC 920 TATGTTCTTA 930 ATAACCTCAA 940 GAGACATTTC 950 TTGTACTTAC 960 ATTATATCTA

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FIG. 1A

550	GAGGTGAAAA	560	ATGTTTATTT	570	CAAAAAATTT	580	ATACCTTTTA	590	TGAATTCTCT	600	TGGACTTGTA
610	CTCCACTTTT	620	TACAAATAAA	630	GTTTTTAAAA	640	TATGGAAAAT	650	ACTTAAGAGA	660	ACCTGAACAT
	ACATCTAATG		GACTTCCAGA		GGTGAAAAAT		CTTCTAAAC		GATACGAAGA		AATTATCTT
	TGTAGATTAC		CTGAAGGTCT		CCAACCTTTA		GAAAGATTG		CTATGCTTCT		TTAAATAGAA
670	AAAAATAAAG	680	ATCTAGATGC	690	AAGATTATTT	700	TTGGATCATG	710	ATAAAACTCT	720	TCAGACTGAT
	TTTTTTATTC		TAGATCTACG		TTCTAATAAA		AACCTAGTAC		TATTTTGAGA		AGCTGACTA
730	TCTATAGACA	740	GTTTTGAAAC	750	ACAGAGAACA	760	CCACGAAAAA	770	GTAACCTTGA	780	TGAAGAGGTG
	AGATATCTGT		CAAAACTTTG		TGCTCTTGT		GGTGCTTTT		CATTGGAAC		ACTTCTCCAC
790	AATGTAATTC	800	CTCCACACAC	810	TCCAGTTAGG	820	ACTGTTATGA	830	ACACTATCCA	840	ACAATTAATG
	TTACATTAAAG		GAGGTGTGTG		AGGTCAATCC		TGACAATACT		TGTGATAGGT		TGTTAATTAC
850	ATGATTTTAA	860	ATTCAGCAAG	870	TGATCAACCT	880	TCAGAAAAATC	890	TGATTTCCTA	900	TTTTAAACAAC
	TACTAAAAAT		TAAAGTCGTC		ACTAGTTGGA		AGTCTTTTAG		ACTAAAGGAT		AAAATTGTTG
910	TGCACAGTGA	920	ATCCAAAAAG	930	AAGTATACTG	940	AAAAGAGTGA	950	AGGATATAGG	960	ATACATCTTT
	ACGTGTCAC		TAGGTTTTCT		TTCATATGAC		TTTTTCTCACT		TCCTATATCC		TATGTAGAAA
970	AAAGAGAAAT	980	TTGCTAAAGC	990	TGTGGGACAG	1000	GGTTGTGTCG	1010	AAATTGGATC	1020	ACAGCGATAC
	TTTCTCTTTA		AACGATTTCG		ACACCCCTGTC		CCAACACAGC		TTTAAACCTAG		TGTCGCTATG
1030	AAACTTGGAG	1040	TTCGCTTGTA	1050	TTACCGAGTA	1060	ATGGAATCCA	1070	TGCTTAAATC	1080	AGAAGAAGAA
	TTTGAACCTC		AAGCGAACAT		AATGGCTCAT		TACCTTAGGT		ACGAATTTAG		TCTTCTTCTT

FIG. 1B

1090	CGATTATCCA	1100	TTCAAAATT	1110	TAGCAAACTT	1120	CTGAATGACA	1130	ACATTTTTCA	1140	TATGTCTTTA
	GCTAATAGGT		AAGTTTTTAA		ATCGTTTGAA		GACTTACTGT		TGTAAAAAGT		ATACAGAAAT
1150	TTGGCGTGCG	1160	CTCTTGAGGT	1170	TGTAATGGCC	1180	ACATATAGCA	1190	GAAGTACATC	1200	TCAGAAATCTT
	AACCGCACGC		GAGAACTCCA		ACATTACCGG		TGTATATCGT		CTTCAATGTAG		AGTCTTAGAA
1210	GATTCTGGAA	1220	CAGATTTGTC	1230	TTTCCCATGG	1240	ATTCTGAATG	1250	TGCTTAATTT	1260	AAAAGCCCTTT
	CTAAGACCTT		GTCATAACAG		AAAGGTACC		TAAGACTTAC		ACGAATTAAA		TTTTTCGGAAA
1270	GATTTTTACA	1280	AAGTGATCGA	1290	AAGTTTTATC	1300	AAAGCAGAAG	1310	GCAACTTGAC	1320	AAGAGAAATG
	CTAAAAAATGT		TTCACCTAGCT		TTCAAAATAG		TTTTCGTCCTC		CGTTGAACTG		TTCTCTTTAC
1330	ATAAAACATT	1340	TAGAACGATG	1350	TGAACATCGA	1360	ATCATGGAAT	1370	CCCTTGCAATG	1380	GCTCTCAGAT
	TATTTTGTA		ATCTTGCTAC		ACTTGTAGCT		TAGTACCTTA		GGGAACGTAC		CGAGAGTCTA
1390	TCACCTTTAT	1400	TTGATCTTAT	1410	TAAACAATCA	1420	AAGGACCGAG	1430	AAGGACCAAC	1440	TGATCACCTT
	AGTGGAAATA		AACTAGAATA		ATTGTGTAGT		TTCCCTGGCTC		TTCCTGGTTG		ACTAGTGGAA
1450	GAATCTGCTT	1460	GTCCTCTTAA	1470	TCTTCCTCTC	1480	CAGAAATAATC	1490	ACACTGCAGC	1500	AGATATGTAT
	CTTAGACGAA		CAGGAGAAAT		AGAAGGAGAG		GTCTTATTAG		TGTGACGTCG		TCTATACATA
1510	CTTTCTCCTG	1520	TAAGATCTCC	1530	AAAGAAAAAA	1540	GTTCAACTA	1550	CGCGTGTA	1560	TTCTACTGCA
	GAAAGAGGAC		ATTCTAGAGG		TTTCTTTTTT		CCAAGTTGAT		GCGCACTTT		AAGATGACGT
1570	AATGCAGAGA	1580	CACAAGCAAC	1590	CTCAGCCTTC	1600	CAGACCCAGA	1610	AGCCATTGAA	1620	ATCTACCTCT
	TTACGTCTCT		GTGTTTCGTTG		GAGTCGGAAG		GTCTGGGTCT		TCGGTAACTT		TAGATGGAGA

FIG. 1C

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1630	CTTTCACGTG	1640	TTTATAAAAA	1650	AGTGATCGG	1660	CTAGCCTATC	1670	TCCGGGCTAAA	1680	TACACTTTGT
1690	GAAAGTGACA	1700	AAATATTTTT	1710	TCACATAGCC	1720	GATCGGATAG	1730	AGGCCGATTT	1740	ATGTGAAACA
1750	GAACGCCCTC	1760	TGCTGAGCA	1770	CCCAGAATTA	1780	GAACATATCA	1790	TCTGGACCCCT	1800	TTTCCAGCAC
1810	CTTGCGGAAG	1820	ACAGACTCGT	1830	GGGTCTTAAT	1840	CTTGATATAGT	1850	AGACCTGGGA	1860	AAAGGTCGTG
1870	ACCCCTGCAGA	1880	ATGAGTATGA	1890	ACTCATGAGA	1900	GACAGGCATT	1910	TGGACCAAAT	1920	TATGATGTGT
1930	TGGGACGTCT	1940	TACTCATACT	1950	TGAGTACTCT	1960	CTGTCCGTAA	1970	ACCTGGTTTA	1980	ATACTACACA
1990	TCCATGTATG	2000	GCATATGCAA	2010	AGTGAAGAAT	2020	ATAGACCTTA	2030	AATTCAAAAT	2040	CATTGTAACA
2050	AGGTACATAC	2060	CGTATACGTT	2070	TCACTTCTTA	2080	TATCTGGAAT	2090	TTAAGTTTAA	2100	GTAACATTGT
2110	GCATACAAGG	2120	ATCTTCCTCA	2130	TGCTGTTTCA	2140	GAGACATTCA	2150	AACGTGTTTT	2160	GATCAAAGAA
2170	CGTATGTTCC	2180	TAGAAGGAGT	2190	ACGACAAGTC	2200	CTCTGTAAGT	2210	TTGCACAAA	2220	CTAGTTTCTT
2230	GAGGAGTATG	2240	ATTCTATTAT	2250	AGTATTCTAT	2260	AACTCGGTCT	2270	TCATGCAGAG	2280	ACTGAAAACA
2290	CTCCTCATAC	2300	TAAGATAATA	2310	TCATAAGATA	2320	TTGAGCCAGA	2330	AGTACGTCTC	2340	TGACTTTTGT
2350	AATATTTTGC	2360	AGTATGCTTC	2370	CACCAGGCCC	2380	CCTACCTTGT	2390	CACCAATACC	2400	TCACATTCCCT
2410	TTATAAAACG	2420	TCATACGAAG	2430	GTGGTCCGGG	2440	GGATGGAACA	2450	GTGGTTATGG	2460	AGTGTAAGGA
2470	CGAAGCCCCTT	2480	ACAAGTTTCC	2490	TAGTTCACCC	2500	TTACGGATTC	2510	CTGGAGGGAA	2520	CATCTATATT
2530	GCTTCGGGAA	2540	TGTTCAAAGG	2550	ATCAAAGTGGG	2560	AATGCCCTAAG	2570	GACCTCCCTT	2580	GTAGATATAA
2590	TCACCCCCTGA	2600	AGAGTCCATA	2610	TAAAATTTC	2620	GAAGGTCTGC	2630	CAACACCAAC	2640	AAAAATGACT
2650	AGTGGGGACT	2660	TCTCAGGTAT	2670	ATTTTAAAGT	2680	CTTCCAGACG	2690	GTTGTGGTTG	2700	TTTTTACTGA

FIG. 1D

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2170	2180	2190	2200	2210	2220
CCAAAGATCAA	GAATCTTAGT	ATCAATTGGT	GAATCATTCG	GGACTTCTGA	GAAGTTCCAG
GGTTCTAGTT	CTTAGAATCA	TAGTTAACCA	CTTAGTAAGC	CCTGAAGACT	CTTCAAGGTC
2230	2240	2250	2260	2270	2280
AAAATAAATC	AGATGGTATG	TAACAGCGAC	CGTGTGCTCA	AAAGAAAGTGC	TGAAGGAAGC
TTTTATTAG	TCTACCATAC	ATTGTCGCTG	GCACACGAGT	TTTCTTCACG	ACTTCCTTCG
2290	2300	2310	2320	2330	2340
AACCCCTCCTA	AACCACTGAA	AAAACCTACGC	TTTGATATTG	AAGGATCAGA	TGAAGCAGAT
TTGGGAGGAT	TTGGTGACTT	TTTTGATGCG	AAACTATAAC	TTCCCTAGTCT	ACTTCGTCTA
2350	2360	2370	2380	2390	2400
GGAAGTAAAC	ATCTCCCAGG	AGAGTCCAAA	TTTCAGCAGA	AACTGGCAGA	AATGACTTCT
CCTTCATTG	TAGAGGGTCC	TCTCAGGTTT	AAAGTCGTCT	TTGAÇCGTCT	TTACTGAAGA
2410	2420	2430	2440	2450	2460
ACTCGAACAC	GAATGCCAAA	GCAGAAAATG	AATGATAGCA	TGGATACCTC	AAACAAGGAA
TGAGCTTGTG	CTTACGTTTT	CGTCTTTTAC	TTACTATCGT	ACCTATGGAG	TTTGTTCCCT
2470	2480	2490	2500	2510	2520
GAGAAATGAG	GATCTCAGGA	CCTTGGTGGA	CACTGTGTAC	ACCTCTGGAT	TCATTGTCTC
CTCTTTACTC	CTAGAGTCCT	GGAACCACTT	GTGACACATG	TGGAGACCTA	AGTAACAGAG
2530	2540	2550	2560	2570	2580
TCACAGATGT	GACTGTATAA	CTTTCCCAGG	TTCTGTTTAT	GGCCACATTT	AATATCTTCA
AGTGTCTACA	CTGACATATT	GAAAGGGTCC	AAGACAAATA	CCGGTGTAATA	TTATAGAAGT
2590	2600	2610	2620	2630	2640
GCTCTTTTGG	TGGATATAAA	ATGTGCAGAT	GCAATTGTTT	GGGTGATTCC	TAAGCCACTT
CGAGAAAAC	ACCTATATTT	TACACGTCTA	CGTTAACAAA	CCCACTAAGG	ATTCCGGTAA
2650	2660	2670	2680	2690	2700
GAAATGTTAG	TCATTGTTAT	TTATACAAGA	TTGAAAATCT	TGTGTAATC	CTGCCATTTA
CTTTACAATC	AGTAACAATA	AATATGTTCT	AACTTTTAGA	ACACATTTAG	GACGGTAAAT

FIG. 1E

2710	2720	2730	2740	2750	2760
AAAAGTTGTA	GCAGATTGTT	TCCTCTTCCA	AAGTAAATTT	GCTGTGCTTT	ATGGATAGTA
TTTTCACACAT	CGTCTAACAA	AGGAGAAGGT	TTCAATTTTAA	CGACACGAAA	TACCTATCAT
2770	2780	2790	2800	2810	2820
AGAATGGCCC	TAGAGTGGGA	GTCCTGATAA	CCCAGGCCCTG	TCTGACTACT	TTGCCCTTCTT
TCTTACCGGG	ATCTCACCCCT	CAGGACTATT	GGTCCGGAC	AGACTGATGA	AACGGAAGAA
2830	2840	2850	2860	2870	2880
TTGTAGCATA	TAGGTGATGT	TTGCTCTTGT	TTTTATTAAAT	TTATATGTAT	ATTTTTTTAA
AACATCGTAT	ATCCACTACA	AACGAGAACA	AAAATAATTA	AATATACATA	TAAATAAATT
2890	2900	2910	2920	2930	2940
TTTAACATGA	ACACCCTTAG	AAAATGTGTC	CTATCTATCT	TCCAAATGCA	ATTTGATTGA
AAATTGTACT	TGTGGGAATC	TTTTACACAG	GATAGATAGA	AGGTTTACGT	TAAACTAACT
2950	2960	2970	2980	2990	3000
CTGCCCATTC	ACCAAATAA	TCCTGAACTC	TTCTGCAAAA	ATGGATATTA	TTAGAAATTA
GACGGGTAAG	TGGTTTAAAT	AGGACTTGAG	AAGACGTTTT	TACCTATAAT	AATCTTTAAT
3010	3020	3030	3040	3050	3060
GAAAAAATTT	ACTAATTTTA	CACATTAGAT	TTTTATTTTAC	TATTGGAATC	TGATATACTG
CTTTTTTTAA	TGATTAAAT	GTGTAATCTA	AAATAAAATG	ATAACCTTAG	ACTATATGAC
3070	3080	3090	3100	3110	3120
TGTGCTTGTT	TTATAAAATTT	TTGCTTTTAA	TTAAATAAAA	GCTGGAAGCA	AAGTATAACC
ACACGAACAA	AATATTTTAA	AACGAAAATTT	AATTTATTTT	CGACCTTCGT	TTCATATTTG
3130	3140	3150	3160	3170	3180
ATATGATACT	ATCATACTAC	TGAAACACAGAT	TTCATACCTC	AGAATGTAAA	AGAACTTACT
TATACTATGA	TAGTATGATG	ACTTTGTCTA	AAGTATGGAG	TCTTACATTT	TCTTGAATGA
3190	3200	3210	3220	3230	
GATTATTTTC	TTCATCCAAC	TTATGTTTTT	AAATGAGGAT	TATTGATAGT	GG 3'
CTAATAAAAG	AAGTAGGTTG	AATACAAAAA	TTTACTCCTA	ATAACTATCA	<u>CCCTAG</u> 5'

FIG. 1F

GATCCCCGA CCTAGATGAG ATG TCG TTC ACT TTT ACT GAG CTA CAG AAA	48
Met Ser Phe Thr Phe Thr Glu Leu Gln Lys	10
AAC ATA GAA ATC AGT GTC CAT AAA TTC TTT AAC TTA CTA AAA GAA ATT	96
Asn Ile Glu Ile Ser Val His Lys Phe Phe Asn Leu Leu Lys Glu Ile	26
GAT ACC AGT ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG	144
Asp Thr Ser Thr Lys Val Asp Asn Ala Met Ser Arg Leu Leu Lys Lys	42
TAT GAT GTA TTG TTT GCA CTC TTC AGC AAA TTG GAA AGG ACA TGT GAA	192
Tyr Asp Val Leu Leu Phe Ala Leu Phe Ser Lys Leu Glu Arg Thr Cys Glu	58
CTT ATA TAT TTG ACA CAA CCC AGC AGT TCG ATA TCT ACT GAA ATA AAT	240
Leu Ile Tyr Leu Thr Gln Pro Ser Ser Ser Ile Ser Thr Glu Ile Asn	74
TCT GCA TTG GTG CTA AAA GTT TCT TGG ATC ACA TTT TTA TTA GCT AAA	288
Ser Ala Leu Val Leu Lys Val Ser Trp Ile Thr Phe Leu Leu Ala Lys	90
GGG GAA GTA TTA CAA ATG GAA GAT GAT CTG GTG ATT TCA TTT CAG TTA	336
Gly Glu Val Leu Leu Gln Met Glu Asp Asp Leu Val Ile Ser Phe Gln Leu	106
ATG CTA TGT GTC CTT GAC TAT TTT ATT AAA CTC TCA CCG CCC ATG TTG	384
Met Leu Cys Val Leu Asp Tyr Phe Ile Lys Leu Ser Pro Pro Met Leu	122
CTC AAA GAA CCA TAT AAA ACA GCT GTT ATA CCC ATT AAT GGT TCA CCT	432
Leu Lys Glu Pro Tyr Lys Thr Ala Val Ile Pro Ile Asn Gly Ser Pro	138
CGA ACA CCC AGG CGA GGT CAG AAC AGG AGT GCA CGG ATA GCA AAA CAA	480
Arg Thr Pro Arg Arg Gly Gln Asn Arg Ser Ala Arg Ile Ala Lys Gln	154

FIG. 2A

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CTA GAA AAT GAT ACA AGA ATT ATT GAA GTT CTC TGT AAA GAA CAT GAA	528
Leu Glu Asn Asp Thr Arg Ile Ile Glu Val Leu Cys Lys Glu His Glu	170
TGT AAT ATA GAT GAG GTG AAA AAT GTT TAT TTC AAA AAT TTT ATA CCT	576
Cys Asn Ile Asp Glu Val Lys Asn Val Tyr Phe Lys Asn Phe Ile Pro	186
TTT ATG AAT TCT CTT GGA CTT GTA ACA TCT AAT GGA CTT CCA GAG GTT	624
Phe Met Asn Ser Leu Gly Leu Val Thr Ser Asn Gly Leu Pro Glu Val	202
GAA AAT CTT TCT AAA CGA TAC GAA GAA ATT TAT CTT AAA AAT AAA GAT	672
Glu Asn Leu Ser Lys Arg Tyr Glu Glu Ile Tyr Leu Lys Asn Lys Asp	218
CTA GAT GCA AGA TTA TTT TTG GAT CAT GAT AAA ACT CTT CAG ACT GAT	720
Leu Asp Ala Arg Leu Phe Leu Asp His Asp Lys Thr Leu Gln Thr Asp	234
TCT ATA GAC AGT TTT GAA ACA CAG AGA ACA CCA CGA AAA AGT AAC CTT	768
Ser Ile Asp Ser Phe Glu Thr Gln Arg Thr Pro Arg Lys Ser Asn Leu	250
GAT GAA GAG GTG AAT GTA ATT CCT CCA CAC ACT CCA GTT AGG ACT GTT	816
Asp Glu Glu Val Asn Val Ile Pro Pro His Thr Pro Val Arg Thr Val	266
ATG AAC ACT ATC CAA CAA TTA ATG ATG ATT TTA AAT TCA GCA AGT GAT	864
Met Asn Thr Ile Gln Gln Leu Met Met Ile Leu Asn Ser Ala Ser Asp	282
CAA CCT TCA GAA AAT CTG ATT TCC TAT TTT AAC AAC TGC ACA GTG AAT	912
Gln Pro Ser Glu Asn Leu Ile Ser Tyr Phe Asn Asn Cys Thr Val Asn	298
CCA AAA GAA AGT ATA CTG AAA AGA GTG AAG GAT ATA GGA TAC ATC TTT	960
Pro Lys Glu Ser Ile Leu Lys Arg Val Lys Asp Ile Gly Tyr Ile Phe	314

FIG. 2B

AAA GAG AAA TTT GCT AAA GCT GTG GGA CAG GGT TGT GTC GAA ATT GGA 1008
 Lys Glu Lys Phe Ala Lys Ala Val Gly Gln Gly Cys Val Glu Ile Gly 330

TCA CAG CGA TAC AAA CTT GGA GTT CGC TTG TAT TAC CGA GTA ATG GAA 1056
 Ser Gln Arg Tyr Lys Leu Gly Val Arg Leu Tyr Tyr Arg Val Met Glu 346

TCC ATG CTT AAA TCA GAA GAA GAA CGA TTA TCC ATT CAA AAT TTT AGC 1104
 Ser Met Leu Lys Ser Ser Glu Glu Glu Arg Arg Leu Ser Ile Gln Asn Phe Ser 362

AAA CTT CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT 1152
 Lys Leu Leu Asn Asp Asn Ile Phe His Met Ser Ser Leu Leu Ala Cys Ala 378

CTT GAG GTT GTA ATG GCC ACA TAT AGC AGA AGT ACA TCT CAG AAT CTT 1200
 Leu Glu Val Val Met Ala Thr Tyr Ser Ser Arg Ser Thr Ser Gln Asn Leu 394

GAT TCT GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG AAT GTG CTT AAT 1248
 Asp Ser Gly Thr Asp Leu Ser Phe Phe Pro Trp Ile Leu Asn Val Leu Asn 410

TTA AAA GCC TTT GAT TTT TAC AAA GTG ATC GAA AGT TTT ATC AAA GCA 1296
 Leu Lys Ala Phe Asp Phe Tyr Lys Val Ile Glu Ser Phe Ile Lys Ala 426

GAA GGC AAC TTG ACA AGA GAA ATG ATA AAA CAT TTA GAA CGA TGT GAA 1344
 Glu Gly Asn Leu Leu Thr Arg Glu Met Ile Lys His Leu Glu Arg Cys Glu 442

CAT CGA ATC ATG GAA TCC CTT GCA TGG CTC TCA GAT TCA CCT TTA TTT 1392
 His Arg Ile Met Glu Ser Leu Ala Trp Leu Ser Asp Ser Pro Leu Phe 458

GAT CTT ATT AAA CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT 1440
 Asp Leu Ile Lys Gln Ser Lys Asp Arg Glu Gly Pro Thr Asp His Leu 474

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FIG. 2C

GAA TCT GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT AAT CAC ACT GCA	1488
Glu Ser Ala Cys Pro Leu Asn Leu Pro Leu Gln Asn His Thr Ala	490
GCA GAT ATG TAT CTT TCT CCT GTA AGA TCT CCA AAG AAA GGT TCA	1536
Ala Asp Met Tyr Leu Ser Pro Val Arg Ser Pro Lys Lys Gly Ser	506
ACT ACG CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA CAA GCA ACC TCA	1584
Thr Thr Arg Val Asn Ser Thr Ala Asn Ala Glu Thr Gln Ala Thr Ser	522
GCC TTC CAG ACC CAG AAG CCA TTG AAA TCT ACC TCT CTT TCA CTG TTT	1632
Ala Phe Gln Thr Gln Lys Pro Leu Lys Ser Thr Ser Leu Ser Leu Phe	538
TAT AAA AAA GTG TAT CCG CTA GCC TAT TAT Tyr Leu Ala Tyr Leu Cys	1680
Tyr Lys Lys Val Tyr Arg Leu His Pro Glu Leu Arg Leu Thr Leu Cys	554
GAA CGC CTT CTG TCT GAG CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC	1728
Glu Arg Leu Leu Ser Glu His Pro Glu Leu Glu His Ile Ile Trp Thr	570
CTT TTC CAG CAC ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG	1776
Leu Phe Gln His Thr Leu Gln Asn Glu Tyr Glu Leu Met Arg Asp Arg	586
CAT TTG GAC CAA ATT ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG	1824
His Leu Asp Gln Ile Met Met Cys Ser Met Tyr Gly Ile Cys Lys Val	602
AAG AAT ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC AAG GAT	1872
Lys Asn Ile Asp Leu Lys Phe Lys Ile Ile Val Thr Ala Tyr Lys Asp	618
CTT CCT CAT GCT GTT CAG GAG ACA TTC AAA CGT GTT TTG ATC AAA GAA	1920
Leu Pro His Ala Val Gln Glu Thr Phe Lys Arg Val Leu Ile Lys Glu	634

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FIG. 2D

GAG	GAG	TAT	GAT	TCT	ATT	ATA	GTA	TTC	TAT	AAC	TCG	GTC	TTC	ATG	CAG	1968
Glu	Glu	Tyr	Asp	Ser	Ile	Ile	Val	Phe	Tyr	Asn	Ser	Val	Phe	Met	Gln	650
AGA	CTG	AAA	ACA	AAT	ATT	TTG	CAG	TAT	GCT	TCC	ACC	AGG	CCC	CCT	ACC	2016
Arg	Leu	Lys	Thr	Asn	Ile	Leu	Gln	Tyr	Ala	Ser	Thr	Arg	Pro	Pro	Thr	666
TTG	TCA	CCA	ATA	CCT	CAC	ATT	CCT	CGA	AGC	CCT	TAC	AAG	TTT	CCT	AGT	2064
Leu	Ser	Pro	Ile	Pro	His	Ile	Pro	Arg	Ser	Pro	Tyr	Lys	Phe	Pro	Ser	682
TCA	CCC	TTA	CGG	ATT	CCT	GGA	GGG	AAC	ATC	TAT	ATT	TCA	CCC	CTG	AAG	2112
Ser	Pro	Leu	Arg	Ile	Pro	Gly	Gly	Asn	Ile	Tyr	Ile	Ser	Pro	Leu	Lys	698
AGT	CCA	TAT	AAA	ATT	TCA	GAA	GGT	CTG	CCA	ACA	CCA	ACA	AAA	ATG	ACT	2160
Ser	Pro	Tyr	Lys	Ile	Ser	Glu	Gly	Leu	Pro	Thr	Pro	Thr	Lys	Met	Thr	714
CCA	AGA	TCA	AGA	ATC	TTA	GTA	TCA	ATT	GGT	GAA	TCA	TTC	GGG	ACT	TCT	2208
Pro	Arg	Ser	Arg	Ile	Leu	Val	Ser	Ile	Gly	Glu	Ser	Phe	Gly	Thr	Ser	730
GAG	AAG	TTC	CAG	AAA	ATA	AAT	CAG	ATG	GTA	TGT	AAC	AGC	GAC	CGT	GTG	2256
Glu	Lys	Phe	Gln	Lys	Ile	Asn	Gln	Met	Val	Cys	Asn	Ser	Asp	Arg	Val	746
CTC	AAA	AGA	AGT	GCT	GAA	GGA	AGC	AAC	CCT	CCT	AAA	CCA	CTG	AAA	AAA	2304
Leu	Lys	Arg	Ser	Ala	Glu	Gly	Ser	Asn	Pro	Pro	Lys	Pro	Leu	Lys	Lys	762
CTA	CGC	TTT	GAT	ATT	GAA	GGA	TCA	GAT	GAA	GCA	GAT	GGA	AGT	AAA	CAT	2352
Leu	Arg	Phe	Asp	Ile	Glu	Gly	Ser	Asp	Glu	Ala	Asp	Gly	Ser	Lys	His	778
CTC	CCA	GGA	GAG	TCC	AAA	TTT	CAG	CAG	AAA	CTG	GCA	GAA	ATG	ACT	TCT	2400
Leu	Pro	Gly	Glu	Ser	Lys	Phe	Gln	Gln	Lys	Leu	Ala	Glu	Met	Thr	Ser	794

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FIG. 2E

ACT CGA ACA CGA ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC 2448
Thr Arg Thr Arg Met Gln Lys Gln Lys Met Asn Asp Ser Met Asp Thr 810

TCA AAC AAG GAA GAG AAA TGA GGATCTCAGG ACCTTGTTGG ACACTGTGTA 2499
Ser Asn Lys Glu Glu Lys ***

CACCTCTGGA TTCATTGTCT CTCACAGATG TGA CTGTATA ACTTCCCAG GTTCTGTTA 2559

TGGCCACATT TAATATCTTC AGCTCTTTTT GTGGATATAA AATGTGCAGA TGCAATTGTT 2619

TGGGTGATTC CTAAGCCACT TGAAATGTTA GTCATTGTTA TTTATACAAG ATTGAAAATC 2679

TTGTGTAAAT CCTGCCATTT AAAAAGTTGT AGCAGATTGT TTCCTCTTCC AAAGTAAAT 2739

TGCTGTGCTT TATGGATAGT AAGAATGGCC CTAGAGTGGG AGTCCTGATA ACCCAGGCCT 2799

GTCTGACTAC TTTGCCTTCT TTTGTAGCAT ATAGGTGATG TTTGCTCTTG TTTTATTAA 2859
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TTTATATGTA TATTTTTTTA ATTTAACATG AACACCCCTA GAAAATGTGT CCTATCTATC 2919

ATCCAAATGC AATTGTATTG ACTGCCCATT CACCAAAATT ATCCTGAACT CTTCTGCAA 2979

AATGGATATT ATTAGAAAATT AGAAAAAAT TACTAATTTT ACACATTAGA TTTTATTTA 3039

CTATTGGAAT CTGATATACT GTGTGCTTGT TTTATAAAAT TTTGCTTTTA ATTAAATAAA 3099

AGCTGGAAGC AAAGTATAAC CATATGATAC TATCATACTA CTGAAACAGA TTTCATACCT 3159

CAGAAATGTA AAGAACTTAC TGATTATTTT CTTCATCCAA CTTATGTTTT TAAATGAGGA 3219

TTATTGATAG TGG 3232

FIG. 2F

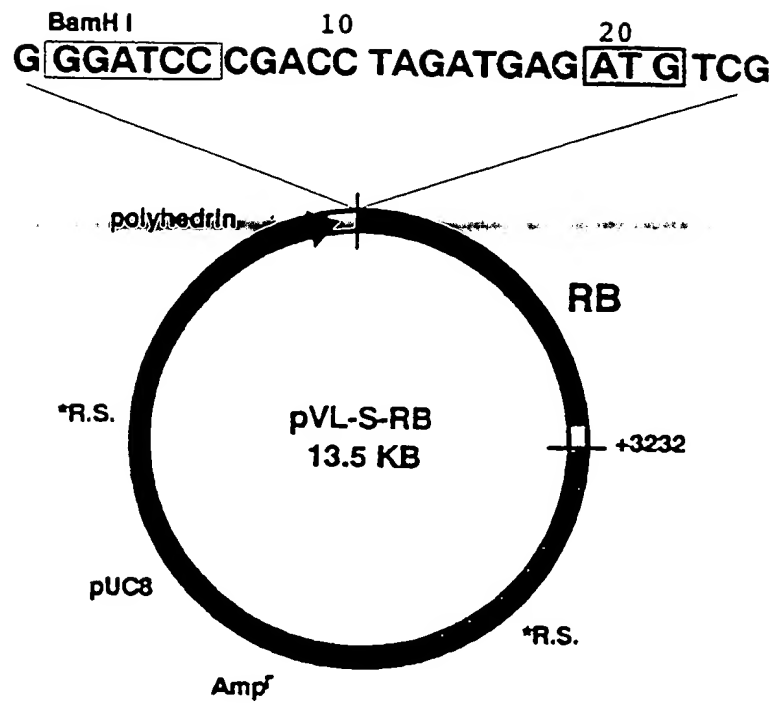


Figure 3.

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FIG. 4C

FIG. 4B

FIG. 4A

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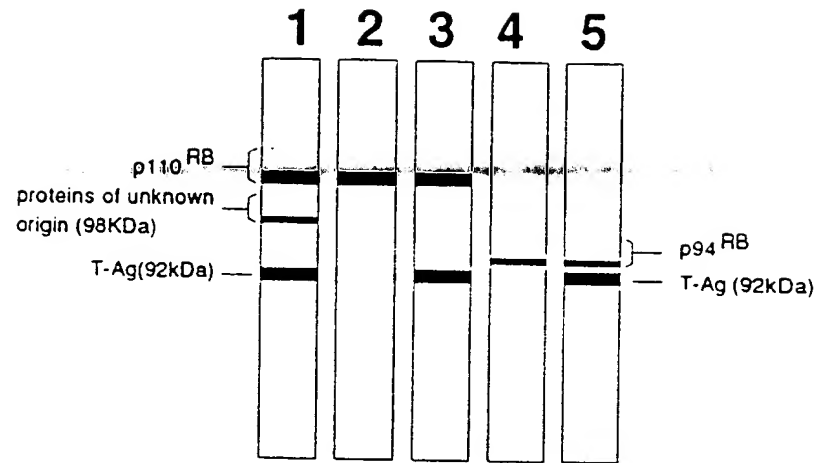
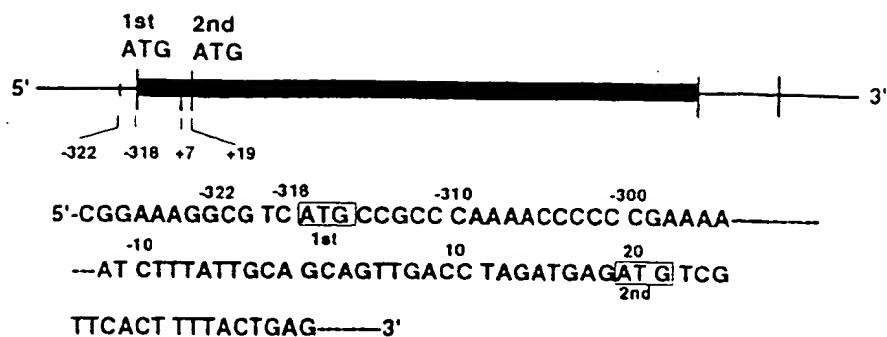


Figure 5.

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A. RB cDNA



B. RB Expression Plasmids

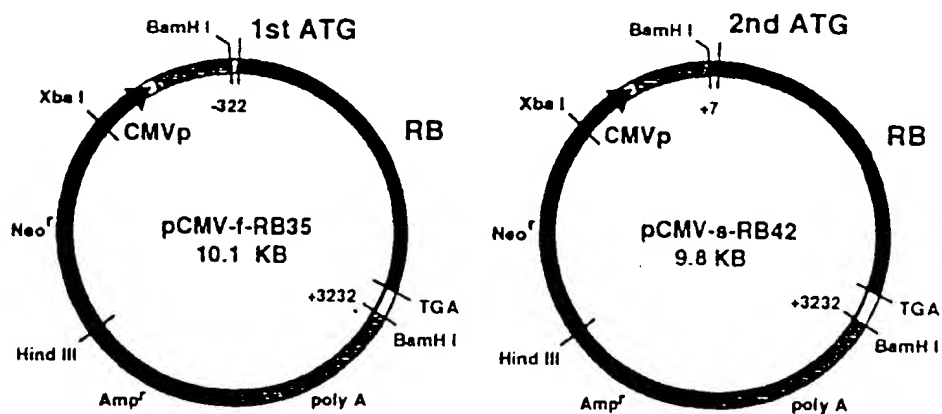


Figure 6.

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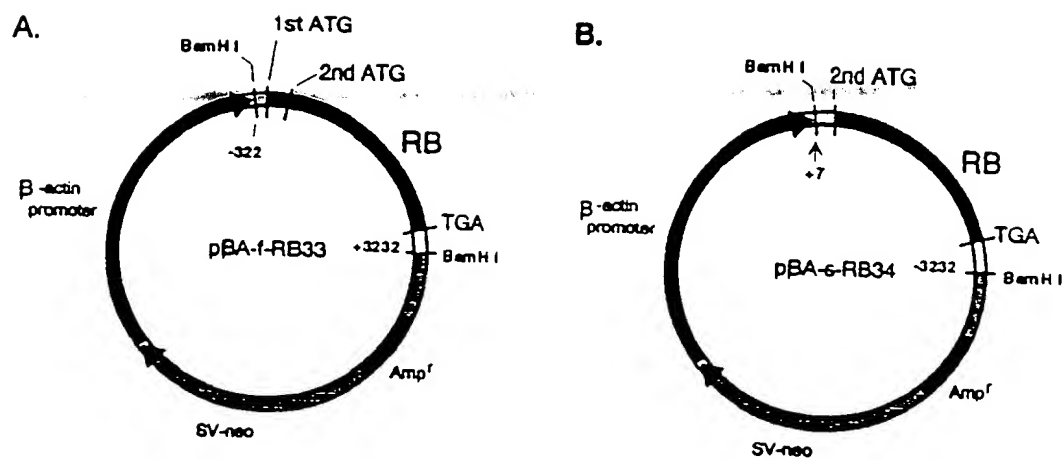


Figure 7.



FIG. 8A

FIG. 8B

FIG. 8C

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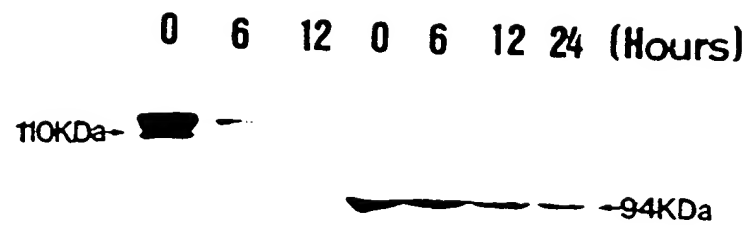


FIG. 9

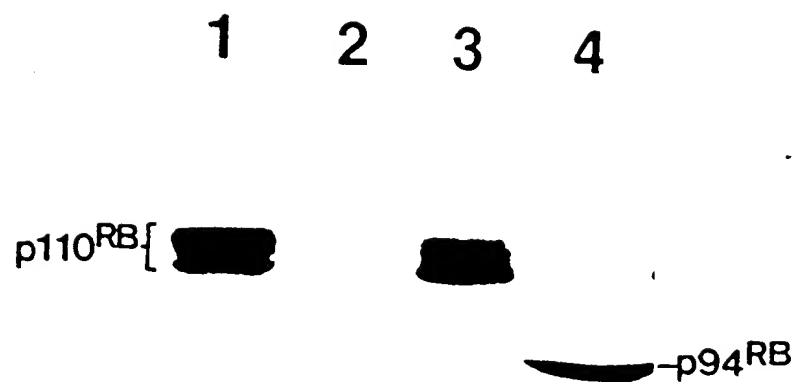


FIG. 10



FIG. 11A

FIG. 11B

FIG. 11C

FIG.12A

FIG.12C



FIG.12B

FIG.12D

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : 435/69.1, 172.3; 514/2, 44; 530/350; 536/24.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3; 514/2, 44; 530/350; 536/24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Volume 249, issued 14 September 1990, E.G. Nabel et al., "Site-specific gene expression in vivo by direct gene transfer into the arterial wall", pages 1285-1288, see entire article.	1-30, 42 and 53
Y	SCIENCE, Volume 235, issued 13 March 1987, W.-H. Lee et al., "Human retinoblastoma susceptibility gene: cloning, identification, and sequence", pages 1394-1399, see entire article.	1-37 AND 40-53
Y	SCIENCE, Volume 254, issued 29 November 1991, R.J. Clem et al., "Prevention of apoptosis by a baculovirus gene during infection of insect cells", pages 1388-1390, see entire article.	36, 37, 40 and 41



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

07 JUNE 1994

Date of mailing of the international search report

JUL 11 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Authorized officer

BRIAN R. STANTON

Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, Volume 360, issued 12 November 1992, D.W. Goodrich et al., "Abrogation by c-myc of G1 phase arrest induces by RB protein but not by p53", pages 177-179, see entire document.	1-35 and 42-53
Y	NATURE, Volume 329, issued 15 October 1987, W.-H. Lee et al., "The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity", pages 642-645, see entire article.	1-37 and 40-53
Y	ONCOGENE, Volume 6, Number 7, issued 1991, H.-J. Xu et al., "Lack of nuclear RB protein staining in G0/middle G1 cells: correlation to changes in total RB protein level", pages 1139-1146, see entire article.	1-37 and 40-53
Y	CANCER RESEARCH, Volume 52, Number 22, issued 15 November 1992, A. Banerjee et al., "Changes in growth and tumorigenicity following reconstitution of retinoblastoma gene function in various human cancer cell types by microcell transfer of chromosome 13", pages 6297-6304, see entire article.	1-37 and 40-53
Y	JOURNAL OF CLINICAL INVESTIGATION, Volume 85, Number 4, issued April 1990, W.F. Benedict et al., "Role of the retinoblastoma gene in the initiation and progression of human cancer", pages 988-993, see entire article.	1-37 and 40-53
Y	THE JOURNAL OF EXPERIMENTAL MEDICINE, Volume 169, number 1, issued 01 January 1989, H. Karasuyama et al., "Autocrine growth and tumorigenicity of interleukin 2-dependent helper T cells transfected with IL-2 gene", pages 13-25, see entire article.	1-37 and 40-53
Y	CELL, Volume 68, issued 10 January 1992, M.A. Rosenfeld et al., "In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium", pages 143-155, see entire article.	1-30, 42 and 53
Y	HUMAN GENE THERAPY, Volume 2, issued 1991, K.W. Culver et al., "Lymphocyte gene therapy", pages 107-109, see entire article.	1-30, 42 and 53
Y	ONCOGENE, Volume 4, Number 4, issued April 1989, A. T'Ang et al., "Genomic organization of the human retinoblastoma gene", pages 401-407, see entire article.	1-37 and 40-53

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ONCOGENE, Volume 4, issued 1989, H.-J. Xu et al., "The retinoblastoma susceptibility gene product: a characteristic pattern in normal cells and abnormal expression in malignant cells", pages 807-812, see entire article.	1-30 and 42-53
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 85, Number 16 issued August 1988, E. Y.-H. P. Lee et al., "Molecular mechanism of retinoblastoma gene inactivation in retinoblastoma cell line Y79", pages 6017-6021, see entire article.	1-35 and 42-53
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 88, Number 12 issued 15 June 1991, R. Takahashi et al., "The retinoblastoma gene functions as a growth and tumor suppressor in human bladder carcinoma cells", pages 5257-5261, see entire article.	1-35 and 42-53
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 84, Number 24, issued December 1987, S.H. Friend et al., "Deletions of a DNA sequence in retinoblastomas and mesenchymal tumors: Organization of the sequence and its encoded protein", pages 9059-9063, see entire article.	1-37 and 40-53
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 84, Number 14 issued July 1987, P. Gunning et al., "A human beta-actin expression vector system directs high-level accumulation of antisense transcripts", pages 4831-4835, see entire article.	1-30, 42 and 53
Y	WO, A, 92/22640 (TAKAHASHI ET AL.) 23 DECEMBER 1992, see entire patent application.	1-37 and 40-53
Y	EP, A, 0,293,266 (FUNG ET AL.) 30 NOVEMBER 1988, see entire patent application.	1-37 and 40-53
Y	WO, A, 90/05180 (LEE ET AL.) 17 MAY 1990, see entire patent application.	1-37 and 40-53
Y	WO, A, 91/15580 (FUNG ET AL.) 17 OCTOBER 1991, see entire patent application.	1-37 and 40-53
Y	NATURE GENETICS, Volume 1, issued August 1992, J.H. Wolfe et al., "Herpesvirus vector gene transfer and expression of beta-glucuronidase in the central nervous system of MPS VII mice", pages 379-384, see entire article.	1-37 and 40-53

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 38 and 39
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 38 and 39 have not been searched because the preambles of said claims recite that methods are claimed and said claims depend from a product claim (claim 32). Therefore it is not possible to determine what process is intended.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A01N 1/00; A61K 31/70, 37/00; C07H 1/00, 3/00, 13/00; C12N 5/00, 15/00; C12P 21/06

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: APS; Biosis Previews; DissAbs; LifeSci; Pascal; HealthPeriodical DB; Medline; Toxline; Cancerlit; Derwent World Patents; Derwent Biotechnology Abs; SciSearch; Genbank

Search terms: p94; retinoblast?; canc?; tumor?; gene?; neoplas?; p110; rb; Xu7/au; Benedict7/au

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I. Claims 1-30 and 53, drawn to DNA encoding the retinoblastoma (Rb) gene and methods of using said DNA for in vivo gene therapy.

Group II. Claims 31-35, drawn to Rb proteins.

Group III. Claims 36, 37, 40 and 41, drawn to a method of making Rb proteins.

Group IV. Claim 42, drawn to ex vivo gene therapy using genes encoding the Rb gene product.

Group V. Claims 43-51, drawn to in vivo therapy using Rb proteins.

Group VI. Claim 52, drawn to ex vivo therapy using Rb proteins.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Any of Groups I or IV-VI and Group III do not relate to a single inventive concept because they are drawn to materially different methods that require divergent areas of search and consideration. For example, the methods of Groups I and IV-VI relate to therapeutic applications which require consideration of appropriate administration routes, selection of suitable adjuvants, methods of targeting therapeutics to appropriate tissue and means of determining efficacy of treatment. Such considerations are not required for methods of making proteins (Group III).

Either of Groups I or V and either of Groups IV or VI do not relate to a single inventive concept because they are drawn to materially different methods that require divergent areas of search and consideration. For example, ex vivo therapy (Groups IV and VI) requires consideration of means of identifying and isolating appropriate target cells, analysis of culture means useful for maintaining said cells ex vivo and consideration of means of administering cellular (Groups IV and VI) versus isolated nucleic acids or proteins (Groups I and V, respectively) compositions.

Either of Groups I or IV and either of Groups V or VI do not relate to a single inventive concept because they are drawn to materially different methods that require divergent areas of search and consideration. For example, the former two methods utilize nucleic acids as therapeutic agents which require consideration of in vivo or intracellular gene expression. Such considerations include selection of suitable promoters and transcriptional and translational signal elements, as well as analysis of the tissue and cell type specificity of said promoter. Such considerations are not required for analysis of protein therapeutic agents (Groups V and VI).

Any of the therapeutic methods of Groups I or IV-VI and the proteins of Group II do not relate to a single inventive concept because the former groups relates to therapeutic applications which require considerations listed above and none of said considerations and corresponding areas of search are required for the analysis of proteins per se as claimed within the invention of Group II.

The proteins of Group II and the methods of making proteins of Group III do not relate to a single inventive concept because proteins may be made by a materially different process from that using recombinant DNA technology

(Group III), such as by direct chemical synthesis and because the methods of Group III require consideration of appropriate gene expression systems and such consideration is not required for analysis of proteins (Group II) per se.